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Potential functionality and digestibility of oryzanol as determined using in vitro cell culture models

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POTENTIAL FUNCTIONALITY AND DIGESTIBILITY OF
ORYZANOL AS DETERMINED USING
IN VITRO CELL CULTURE MODELS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by
Chih-chun Jean Huang
B.S., National Taiwan University, 1994
M.S., Louisiana State University, 1999
August 2003

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DEDICATION

To my parents, Juo-jun Huang and Sung Fan,
who taught me to be responsible and professional in any field,
even what no one minds

ACKNOWLEDGEMENTS

I would like to thank Dr. J. Samuel Godber for giving me an opportunity to learn from him and for his philosophy, guidance and support. He is the best teacher I have ever had. If I become a professor some day, I will wish I could be like him.

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I am grateful for this incredible journey given by God and supported from God, who never did and never will fail me.

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At last, not least, I appreciate my parents, sister and brother, who have encouraged me to pursue my dream and supported me endlessly, and Lai, the love of my life, who has been with me in sunny and rainy days.

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LIST OF ABBREVIATION

AAPH	2,2'-azobis(2-methylpropionamidine) dihydrochloride
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C2BBel	a brush border expressing human colon cell line cloned from the Caco-2 cell line (ATCC HTB-37)
CEase	cholesterol esterase
CPA	bovine pulmonary artery endothelial cell
DMEM	Dulbecco's modified Eagle's medium
FHs74Int	a human normal small intestine cell line
GC/MS	gas chromatography coupled with mass spectrometry
HBSS	Hank's balanced salt solution
HDL	high-density lipoprotein
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
HUV-EC-C	a normal human umbilical vein endothelial cell line
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MTT	[3-(4,5-dimethylthiazol-yl)-2-5-diphenyltetrazolium bromide]
PBS	phosphate buffered saline
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate or sodium lauryl sulfate

SVEC4-10	a mouse endothelial cell line derived by SV40 (strain 4A) transformation of endothelial cells from axillary lymph node vessels
TBA-RS	thiobarbituric acid-reactive substances
tBHP	<i>tert</i> -butyl hydroperoxide
TMCS	trimethylchlorosilane
TMP	1,1,3,3-tetramethoxypropane
TMS	trimethylsilyl group
Tris-HCl	Tris(hydroxymethyl)aminomethane+HCl
VLDL	very low-density lipoprotein

ABSTRACT

A mouse lymphatic endothelial cell (SVEC4-10) model and a human intestinal cell (C2BBel) model *in vitro* were developed and capable to be used to study antioxidant activity, hypocholesterolemic capability and digestibility of γ -oryzanol. The critical and vital parameters in developing these cell models were the emulsion preparation of hydrophobic compounds for cell models, the consistent management of cell culture, and the selection of cell viability detection methods compatible with the cell lines and the test substances. The results showed that, in some situations, γ -oryzanol could present a more effective antioxidant activity than α -tocopherol, in terms of reducing *tert*-butyl hydroperoxide promoted oxidative damage on cellular mitochondrial activity. After 1-hour oxidation, cell viability was 81.8% when incubated with γ -oryzanol, compared to 54.5% with the control and 74.6% with α -tocopherol. The three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate, generally had higher antioxidant activity than γ -oryzanol and among them, 24-methylene cycloartanyl ferulate was found to be relatively more effective and could be more powerful than α -tocopherol. A synergistic antioxidant activity among γ -oryzanol, ferulic acid and α -tocopherol was also found. With regard to the hypocholesterolemic capacity of γ -oryzanol, the results suggested that the intact γ -oryzanol was poorly absorbed by intestinal cells *in vitro*. Therefore, it was speculated that the effect of γ -oryzanol might take place in the lumen of gastrointestinal tract, possibly by means of reducing the micellar solubility of cholesterol and cholesteryl esters and inhibiting cholesterol esterase-facilitated cholesteryl ester hydrolysis. The results showed that preincubation of cholesterol and γ -oryzanol for six hours significantly reduced the cholesterol uptake into cells. γ -Oryzanol also showed a trend towards inhibition of cholesteryl esterase, which is responsible for hydrolyzing cholesteryl esters to free cholesterol

before uptake. The results of *in vitro* digestibility studies of γ -oryzanol showed that the ester bond of γ -oryzanol, especially campesterol ferulate and sitosterol ferulate, was broken down by cholesterol esterase and produced triterpene alcohols or sterols and ferulic acid, which were further degraded. The degradation of γ -oryzanol in the stomach may undergo a different pathway because sterols or triterpene alcohols were also not found after peptic digestion.

CHAPTER 1. INTRODUCTION

While the international scientific research community has recognized the health promoting potential of rice bran for many years, the rice-processing industry in the United States has just recently begun to explore and develop its commercially feasible utilization. In this study, the background of γ -oryzanol and rice bran including sources, analytical methods, toxicity, metabolism and potential functionality was reviewed (Chapter 2).

γ -Oryzanol has been suggested to have potential functionality such as hypocholesterolemic capability and antioxidant activity. The goal of this study was to develop cell culture models as tools to study these two potential aspects of functionality (Chapter 3 and Chapter 4) and digestibility (Chapter 5) of γ -oryzanol. Using cell culture models enables us to speculate and monitor a particular biochemical reaction to a certain cell type in humans in a less expensive and invasive way.

γ -Oryzanol components have been found to have higher antioxidant activity than vitamin E components in some chemical models. One of the objectives was to determine the antioxidant activity of γ -oryzanol and its three major components, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate, and compare them to ferulic acid and α -tocopherol using *tert*-butyl hydroperoxide (tBHP) induced oxidation on an *in vitro* mouse lymphatic endothelial cell (SVEC4-10) model. The critical conditions to develop a cell culture model and the effects of γ -oryzanol incorporation with cells before tBHP oxidation on its antioxidant activity and synergistic antioxidant activity with ferulic acid and α -tocopherol were also discussed.

Some studies have shown that the serum and liver cholesterol levels in rats were lowered after the consumption of rice bran oil or dietary γ -oryzanol feeding. Another objective of this

study was then to explore the hypocholesterolemic effects of γ -oryzanol focusing on cholesterol uptake into human intestinal cells (C2BBe1) *in vitro*. It has been believed that a reduced cholesterol uptake or an increase of fecal cholesterol excretion would correspond to decreased serum total cholesterol levels and the cholesteryl esters in the gastrointestinal lumen should be hydrolyzed by cholesterol esterase before absorption. Hence, the functions of γ -oryzanol on cholesterol uptake into C2BBe1 cells *in vitro* investigated in this study were the effects of γ -oryzanol on reducing micellar solubility of cholesterol and cholesteryl esters and on inhibiting cholesterol esterase-facilitated cholesteryl ester hydrolysis.

Additionally, there has been little information published in regard to the digestibility, the metabolic fate and toxicity of digest products and metabolites of γ -oryzanol in humans. To further understand where the functionality of γ -oryzanol takes place and how it functions relies on the fate of γ -oryzanol after ingestion. Therefore, digestibility of γ -oryzanol with emphasis on peptic and pancreatic digestions and the effect of three pancreatic enzymes, cholesterol esterase, lipase and phospholipase A₂, and the uptake of intact γ -oryzanol into C2BBe1 cells were also studied.

CHAPTER 2. LITERATURE REVIEW

2.1 Rice Bran and Oryzanol

Rice bran is a relatively abundant byproduct of rice milling. Rice bran oil for food use produced commercially in the United States started in 1994, while it has been extensively consumed in many Asian countries. The annual world rice bran oil production is estimated to be less than 800,000 metric tons or about only 1% of all vegetable oils (McCaskill and Zhang, 1999). With the rising awareness of functional food consumption, the demand, value and production are expected to be higher.

A rice kernel consists of 20% hull, 72% endosperm, 6% bran, and 2% germ (Figure 2.1) (Juliano and Bechtel, 1985; Lu and Luh, 1991). Rice bran is the soft germ and several soft layers, pericarp, seed coat, nucellus and aleurone layer, surrounding the hard starchy endosperm. Rice bran contains 15-20% oil, generally the same as soybeans, 12-16% proteins, 34-52% available carbohydrates, 7-11% crude fiber, and 7-10% ash. The typical crude rice bran oil is composed of 68-71% triacylglycerols, 2-3% diacylglycerols, 5-6% monoacylglycerols, 2-3% free fatty acids, 5-7% glycolipids, 3-4% phospholipids (mainly phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol), 2-3% waxes, and 4.2% unsaponifiable fraction (McCaskill and Zhang, 1999; Sayre and Saunders, 1990). Free fatty acids, monoacylglycerols and diacylglycerols are associated with enzymatic hydrolysis. Sterols, existing as free or esterified, are the major portion in the unsaponifiable fraction and classified as 1) 4-desmethylsterols, i.e. normal phytosterols without methyl groups at C-4 position; 2) 4-monomethylsterols and 3) 4,4'-dimethylsterols, also known as triterpene alcohols. Rice bran oil contains more non-triacylglycerol components than most vegetable oils but these are lost during refining processes.

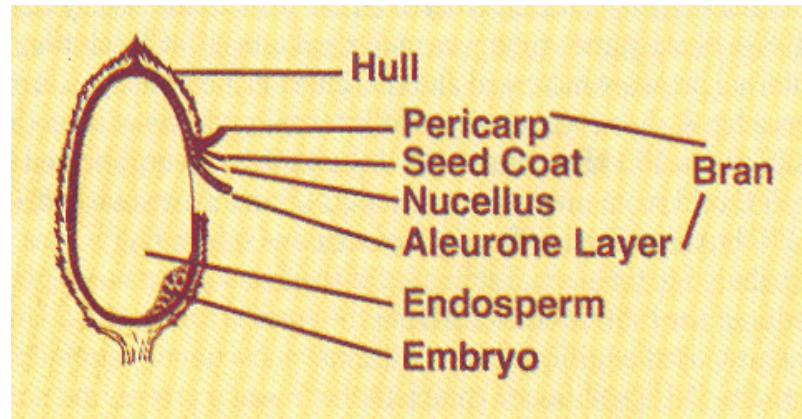


Figure 2.1. Rice kernel structure adapted from Orthoefer, 1996

Oryzanol occurs in the unsaponifiable fraction of rice bran oil and is so named because it was first discovered in rice bran oil (*Oryza Sativa L.*) (Kaneko and Tsuchiya, 1954) and contained a hydroxyl group. Oryzanol was originally considered a single compound but later was determined to be a mixture of ferulic acids esterified with normal sterols or triterpene alcohols, called α -, β - and γ -oryzanol, of which γ -oryzanol has been the most commonly mentioned. The sterol components of γ -oryzanol are primarily campesterol and sitosterol, and the triterpene alcohol components are cycloartenol and 24-methylene cycloartanol (Figure 2.2(b)). The content of γ -oryzanol in rice bran oil can range from 1-3% (Seetharamaiah and Prabhakar, 1986), depending on the rice varieties and analytical methods. The most accessible natural source of γ -oryzanol is rice bran, but some components of γ -oryzanol, mainly sitostanyl ferulate and campestanol ferulate and lesser amounts of sitosteryl ferulate and campesteryl ferulate, can also be found mostly in the inner pericarp of corn, wheat, rye and triticales grains (Seitz, 1989).

2.2 Biological Aspect of γ -Oryzanol

The biological function of γ -oryzanol in the plant has not been clearly described. Since it is a mixture of ferulate esters with sterols or triterpenes, one may view it from two standpoints, triterpenes or sterols and ferulic acid. Triterpenoids can be categorized under the class synonymously named terpenoids, terpenes or isoprenoids, which are various, widespread and numerous natural products derived from a common biosynthetic pathway based on mevalonate as the parent compound, with the important subclass of steroids (Banthorpe, 1991). The general pathway of biosynthesis (Figure 2.3) is enzymatically controlled and starts from acetyl-CoA, to mevalonate, to *2E,6E*-farnesyl pyrophosphate, to squalene, and then to triterpenoids, with a final chair-boat-chair-boat configuration (Bramley, 1997). Phytosterols are further formed from triterpenoids or cycloartenol, which plants apparently use (Connolly and Hill, 1991), involving

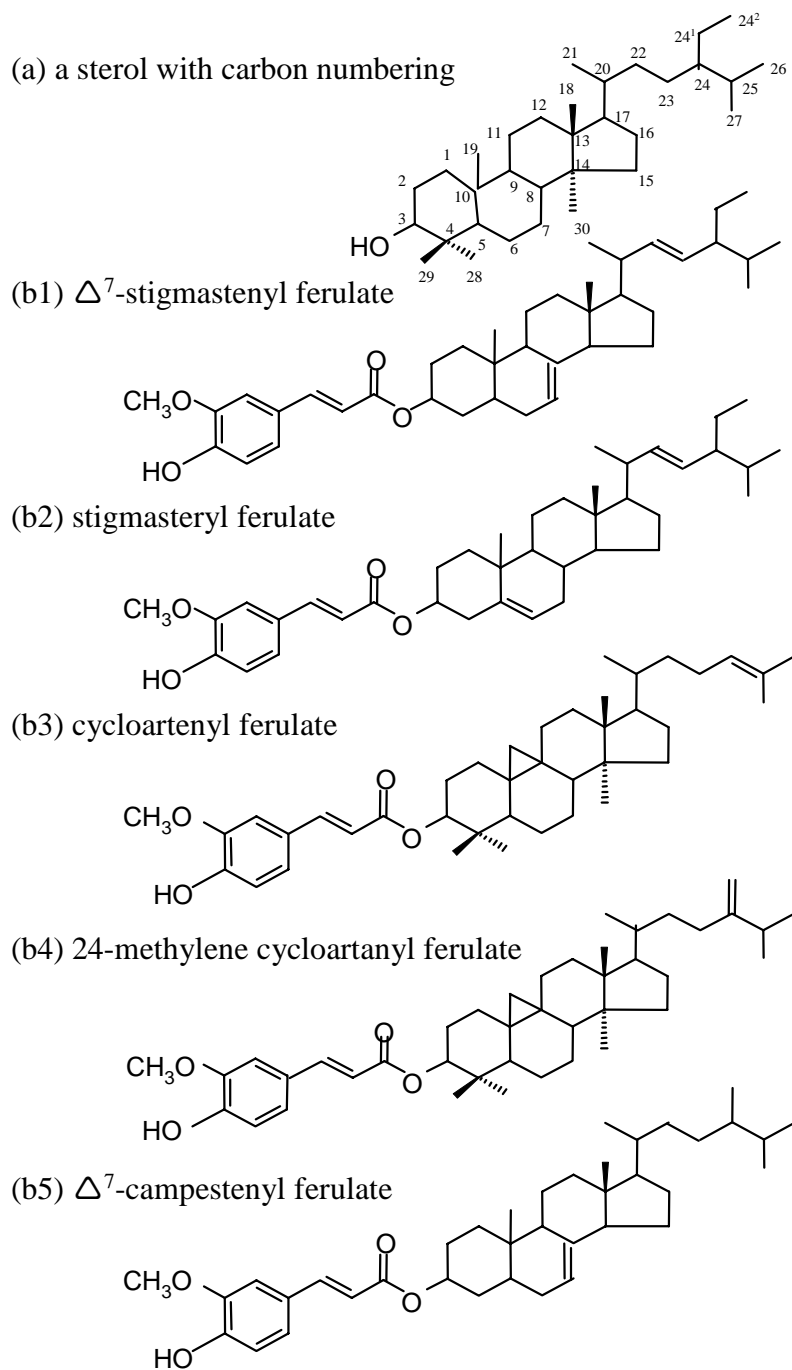


Figure 2.2. Chemical structures of (a) a sterol with carbon numbering, (b1)-(b10) γ -oryzanol components and (c) cholesterol

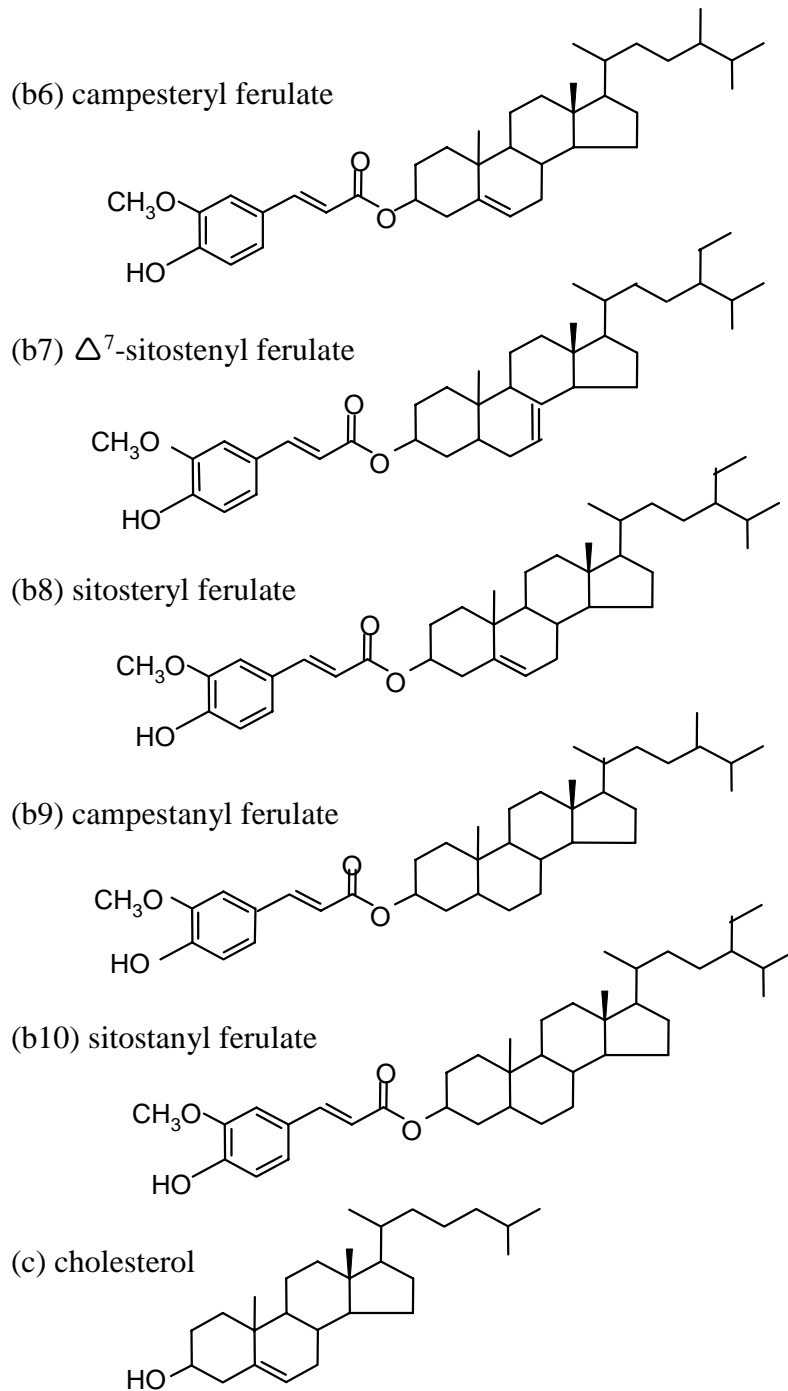
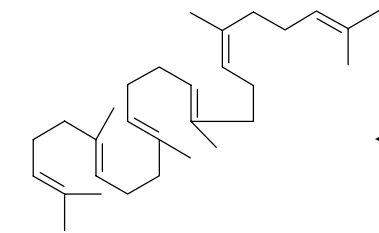


Figure 2.2. Chemical structures of (a) a sterol with carbon numbering, (b1)-(b10) γ -oryzanol components and (c) cholesterol



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the removal of three methyl groups in the sequence C-4, C-14 and then C-4 ((see Figure 2.2(a) for numbering system according to IUPAC-IUB (1989); Bramley (1997); and Goad (1991)). The initial pathway from acetyl-CoA to squalene is common to all organisms, but the sequence of the modifications of the sterol ring system and side chain can differ from species or even in various tissues or during different developmental stages in a plant. Sterol production supposedly occurs in the cytosolic and microsomal compartments of the plant cells.

Phytosterols play a structural role in plant cells as an essential membrane constituent by analogy with cholesterol in animal cells (Goad, 1991), which regulates its fluidity and enhances the mechanical stability of the membrane. The plasma membrane has the highest level of sterols and the greatest ratio of sterol:phospholipid. The endoplasmic reticulum has a lower content and the mitochondrial and the chloroplast membranes contain a small amount of free sterol. Sterols are important for plant growth in two ways. One is for new membrane production by dividing cells and the other is for a 24-ethylsterol such as stigmasterol for specific stimulatory function for cell division to proceed.

On the other hand, ferulic acid is initially converted from phenylalanine and tyrosine by enzymes (Figure 2.4) (Graf, 1992) and much of the ferulic acid occurs as esters in many plants, which may imply the pathway further undergoes conjugation with other molecules. The biological role of ferulic acid has been suggested to crosslink some cell components and lower their availability to hydrolytic degradation by endosperm enzymes and thus inhibit germination. In addition, some studies imply a defensive role of ferulic acid with repellent property and a physiological function in plant mineral metabolism.

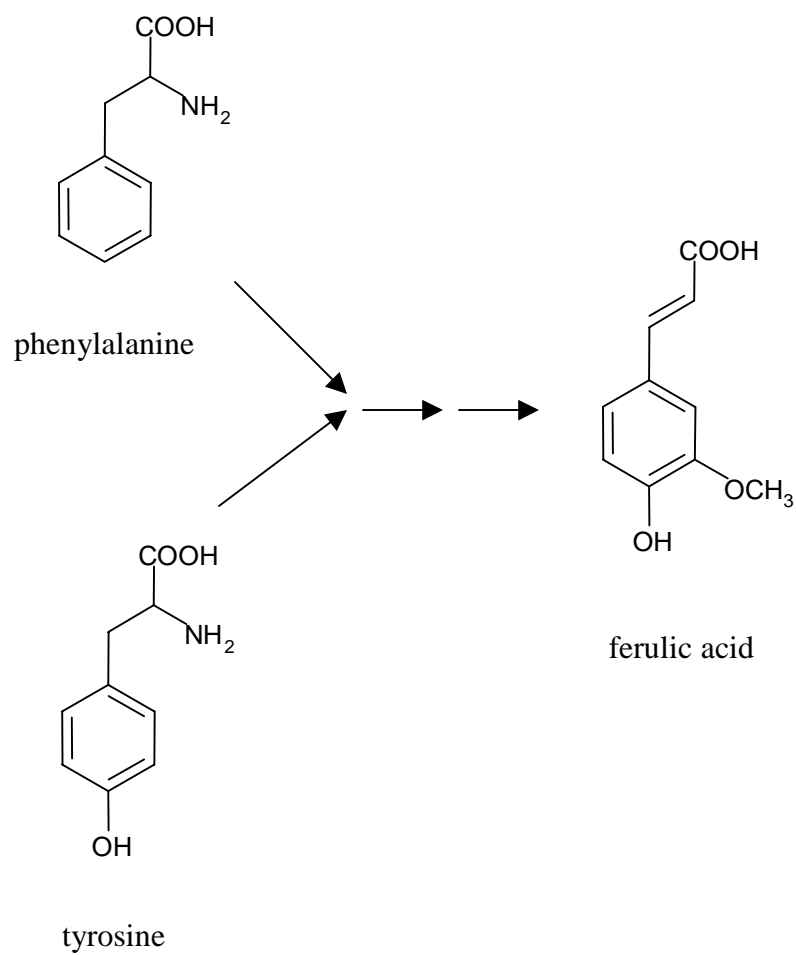


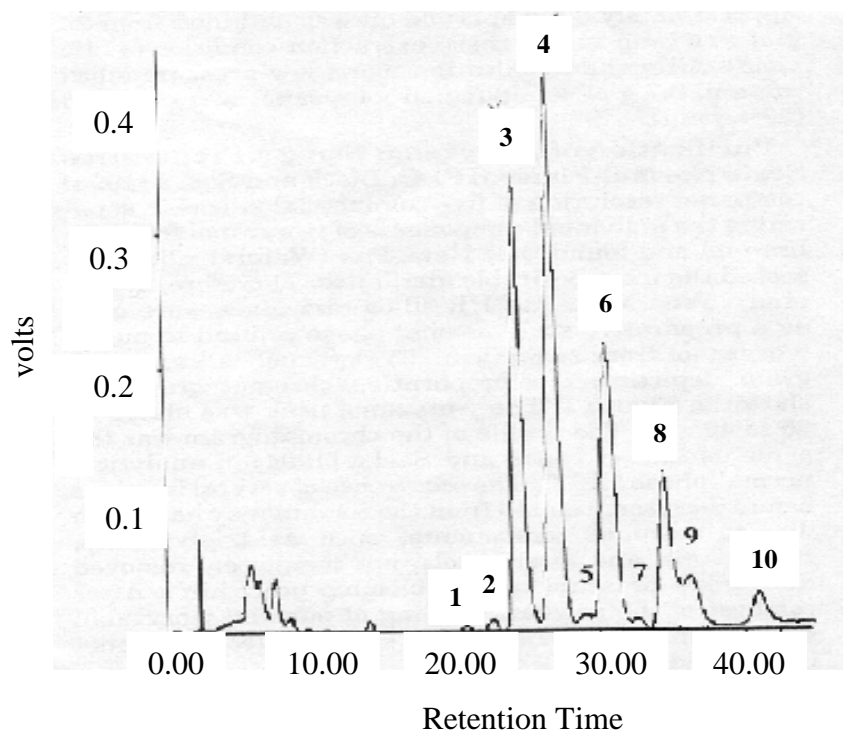
Figure 2.4. Brief metabolic pathways of ferulic acid formation in plants

2.3 Development of γ -Oryzanol Analysis

It has been established that γ -oryzanol is a mixture and the number of individual components found in γ -oryzanol depends on the chromatographic approaches. Normal-phase high performance liquid chromatography (HPLC) used by Diack and Saska, 1994, could only separate γ -oryzanol into two fractions and each fraction contained at least two or more constituents. Identification and quantification of each individual component of γ -oryzanol would be difficult due to incomplete separation. Reverse-phase HPLC, on the other hand, could identify five (Norton, 1995) or six (Evershed *et al.*, 1988; Rogers *et al.*, 1993) individual components of γ -oryzanol. Furthermore, Xu and Godber, 1999, successfully separated and identified ten components of γ -oryzanol from rice bran oil using preparative normal-phase HPLC to concentrate γ -oryzanol and to reduce interfering substances and reverse-phase HPLC to isolate and collect each component (Figure 2.5). These ten components were identified as Δ^7 -stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7 -campestenyl ferulate, campesteryl ferulate, Δ^7 -sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate and sitostanyl ferulate (see Figure 2.2(b) for chemical structures). The three major components among these are cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate.

2.4 Influence of Rice Bran and Rice Bran Oil Processing on γ -Oryzanol

After milling, hydrolytic rancidity by enzymes in the bran occurs rapidly. Methods developed to accomplish stabilization are heating with an extruder or microwave oven, freezing and treating with chemicals. Therefore, after being abraded during rice milling, bran is commonly stabilized by extrusion to inactivate the indigenous lipase and prevent further hydrolysis and the production of free fatty acids and glycerol.



1. Δ^7 -stigmastenyl ferulate,
2. stigmasteryl ferulate,
3. cycloartenyl ferulate,
4. 24-methylenecycloartanyl ferulate,
5. Δ^7 -campestenyl ferulate,
6. campesteryl ferulate,
7. Δ^7 -sitostenyl ferulate,
8. sitosteryl ferulate,
9. campestanyl ferulate, and
10. sitostanyl ferulate

Figure 2.5. HPLC chromatogram of γ -oryzanol from Xu and Godber, 1999

The stability and storability of γ -oryzanol in rice bran after extrusion has been studied by Shin *et al.*, 1997. Rice bran was extruded at temperatures ranging from 110 to 140°C with post extrusion holding times 0, 3, and 6 minutes. γ -Oryzanol, total vitamin E and free fatty acids contents were detected after each treatment at 0 and 7 to 375 days of storage time. It was found that the post extrusion holding time had no effect on hydrolytic stability and the extrusion temperature at 110°C was less effective. The loss of γ -oryzanol and total vitamin E increased when the extrusion temperature increased, which were in a temperature-dependent manner, but the degree of total vitamin E loss was greater than that of γ -oryzanol loss. The total vitamin E content decreased when the holding time increased, while γ -oryzanol content decreased only after 6 minutes of holding time. Additionally, during storage after extrusion, most of the total vitamin E was lost by 210 days of storage, while the degradation rate of γ -oryzanol was constant and more than one third of γ -oryzanol was still present throughout storage. In summary, γ -oryzanol was more stable than vitamin E to the heat of extrusion and decomposition after extrusion during one year of storage at ambient temperature.

After rice bran stabilization, crude rice bran oil is extracted and then refined before human consumption. The process of rice bran oil refining usually involves degumming, neutralization, bleaching, deodorization, and hydrogenation. It has been reported that, in general, the total sterol loss after oil refining may be between 10 to 70%, depending on the type of oil and the processing conditions employed, and the sterol content gradually decreases in each step of the refining process (Kochhar, 1983).

In the neutralization step, the use of alkali such as caustic soda (NaOH) to remove free fatty acids forms soapstock with a significant amount of γ -oryzanol, which is later separated by settling or centrifugation. The soapstock can be reacidified to yield dark acid oil, in which the

free fatty acids can be distilled under high vacuum and leaves γ -oryzanol in the residue called pitch. It has been proposed to recover γ -oryzanol from the inexpensive pitch as a valuable byproduct (Das *et al.*, 1998). In the bleaching step, the purpose is to remove color materials by heating to 85°C and treating with adsorbants such as activated carbon and activated bleaching earth. It has been recorded that partial modification of sterols, i.e. dehydration and oxidation, and deacylation of sterol esters may occur during bleaching. In the deodorization step to remove volatile compounds with undesirable flavors, steam distillation under reduced pressure is usually used with the expectation that nonvolatile off-flavor substances will be thermally degraded, become volatile and be distilled away.

Finally, the hydrogenation process, which is important in the oil industry, allows the liquid oils to be converted into semisolid or solid fats for special applications such as margarine. The process is to add hydrogen to double bonds in the fatty acid chains, which are susceptible to oxidation, and thus also improves the oxidative stability of the oil. The effect of bleaching, deodorization and hydrogenation on γ -oryzanol content in refined oil has not been clearly studied, which may be related to the fact that γ -oryzanol is mainly lost after neutralization. However, since it has been noted that γ -oryzanol has several double bonds and possesses antioxidant activity, if it survives neutralization, γ -oryzanol content may further decrease during these steps where oxidation may occur.

Not only γ -oryzanol but also tocotrienols are lost during each step of rice bran oil refining process, and after processing, up to 90% can be lost. This implies that new refining methods for rice bran oil must be developed and the recoveries of these beneficial ingredients must be well optimized in order to increase the value of rice bran oil.

2.5 Toxicology and Carcinogenicity Studies of γ -Oryzanol

The safety of γ -oryzanol for human consumption was tested by toxicology studies on rats fed a diet containing 10% rice bran oil compared to groundnut oil for three generations (Rukmini, 1988). The chemical composition between these two oils was similar, except the unsaponifiable matter of rice bran oil (4.1%) was higher. The rice bran oil effect on the growth performance, including weight gain and feed efficiency, was not significantly different and the effect on fat absorption and the retention of nitrogen, phosphorus and calcium was not significantly different. Moreover, the toxicological effect of rice bran oil on reproductive performance was evaluated for two matings and three generations. The results indicated that no abnormalities were found, which were comparable to groundnut oil groups on the percentage of conception, birth weight, litter size, weaning weight, preweaning mortality and the number of days taken to deliver from the date of introduction for mating. The mutagenic potential of rice bran oil for deep frying and repeated heating was also found to be negative, which demonstrated remarkable oxidative stability.

Furthermore, the short-term safety of γ -oryzanol was assessed using the Rec assay (bacterial DNA repair test), the Ames test (bacterial reverse mutation test), the rat bone marrow chromosome aberration test, and the metabolic cooperation inhibition test using Chinese hamster V79 cells (Tsushimoto *et al.*, 1991). γ -Oryzanol showed negative responses to all these tests.

Additionally, potential carcinogenesis of γ -oryzanol was studied by feeding mice a diet containing γ -oryzanol up to 2g/kg body weight/day for 78 weeks (Tamagawa *et al.*, 1992b) and feeding rats for 2 years (Tamagawa *et al.*, 1992a). No treatment-related change was found in general condition, food consumption, mortality, organ weight and hematology. Histopathological

examination showed that the tumor incidents were not significantly different between the treated groups and the control groups.

Modifying effects of γ -oryzanol were also studied in a rat wide-spectrum organ carcinogenesis model (Hirose *et al.*, 1991). The three combined carcinogens used were 2,2'-dihydroxy-di-n-propylnitrosamine (1g/kg body weight \times 2 i.p. injections), *N*-ethyl-*N*-hydroxyethylnitrosamine (1.5g/kg body weight \times 2 i.g. administration) and 3,2'-dimethyl-4-aminobiphenyl (75mg/kg body weight \times 3 subcutaneous injections) treatment at intervals of 3 to 4 days. One week after the combined treatment, the animals were fed a basal diet containing 1% γ -oryzanol for 32 weeks and then sacrificed for complete autopsy. The results showed that the γ -oryzanol treatment tended to lower the incidences of pancreatic eosinophilic foci and liver hyperplastic nodule and hepatocellular carcinoma, but increased the incidences of lung adenoma and carcinoma. However, the 1% γ -oryzanol dose given in their study was 100 to 150 times higher than what γ -oryzanol is usually given for medical use ($>300\text{mg/person/day}$). Also, the correlation between γ -oryzanol ingestion and human lung cancer and the interaction with other ingredients in the diet have not been reported in any epidemiological data.

On the other hand, another study of modifying effects on carcinogenesis was demonstrated using a similar animal model (Imaida *et al.*, 1990) but with two different combined carcinogens, 1,2-dimethylhydrazine (40mg/kg body weight \times 3 subcutaneous injections within one week) and 1-methyl-1-nitrosourea (20mg/kg body weight \times 2 i.p. administrations, twice a week for 2 weeks). The animals were then on a diet containing 1% ferulic acid until autopsy at the 52nd week. The combined carcinogens could cause initiation in many organs such as forestomach, glandular stomach, alimentary tract, urinary bladder, kidney, lung, nervous system, mammary gland and hematopoietic system and induce colon carcinomas in rats. The results

showed that 1% ferulic acid had weak inhibitory effect and did not promote lung adenoma and carcinoma as γ -oryzanol did in the study by Hirose *et al.* (1991).

Finally, γ -oryzanol and its four major components, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate and sitosteryl ferulate have been reported to have an inhibitory effect on tumor promotion in two-staged carcinogenesis in mouse skin (Yasukawa *et al.*, 1998). Both the inner and outer surfaces of the ear of each mouse were treated topically with γ -oryzanol and its components at different concentrations, followed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (1 μ g/ear) to induce inflammation. The ear thickness was determined to compare the anti-inflammatory activities. The results showed that, while γ -oryzanol had no significant effect, its four components had inhibitory activities in a concentration-dependent manner. Their 50% inhibitory dose was lower than quercetin, a known tumor inhibitor, similar to indomethacin and higher than hydrocortisone. The inhibitory ratio was higher than quercetin and comparable with indomethacin and hydrocortisone. Furthermore, the TPA promoting effect on papillomas induced by 7,12-di-methylbenz[*a*]anthracene was remarkably inhibited by cycloartenyl ferulate. Esterification of triterpenes with ferulic acid had no influence, but sitosteryl ferulate was found more effective than free sitosterol. Interestingly, the topical application of γ -oryzanol was found to significantly increase sebaceous secretion of the skin, turnover of sebaceous gland cells and peripheral blood flow, which indicated that γ -oryzanol may be useful to repair damaged or dry skin. In fact, γ -oryzanol has been used in skin lotion for its cosmetic and pharmaceutical effects.

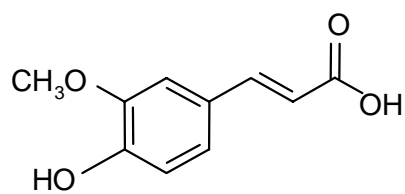
In summary, γ -oryzanol has been approved and commercially available in many countries such as Japan but government and industry in the United States are comparably more conservative. Studies suggest that γ -oryzanol is not carcinogenic to animals and rice bran oil was

safe for human consumption. There have been no reported acute or chronic side effects of γ -oryzanol and the evidence of its beneficial effects in humans is increasing and the market is growing. Thus, extensive and well-designed clinical trials with regard to the potential of γ -oryzanol and ferulic acid as chemopreventors or promoters and the mechanisms for carcinogenic or anticarcinogenic effects are needed.

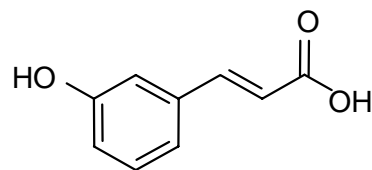
2.6 Digestibility, Absorption and Metabolism of γ -Oryzanol

While γ -oryzanol has been utilized in many countries as food additives and pharmaceuticals, there are only a few studies in English exploring the digestibility of γ -oryzanol in the human gastrointestinal tract. Many of the studies used animal models such as rabbits and rats and they were also done using thin layer chromatography in the period when the analytical method to identify γ -oryzanol and its metabolites was not advanced.

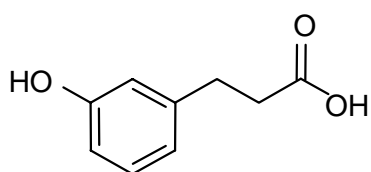
One of the few studies about absorption, metabolism and lymphatic transport of γ -oryzanol - ^{14}C orally administered to rats (50 mg/kg) was by Fujiwara *et al.*, 1983. After 72 hours of administration, 9.8% radioactivity was found in urine and 84.5% was in feces. That found in the urine was not intact γ -oryzanol but identified as the major metabolites such as ferulic acid, dihydroferulic acid, *m*-hydroxyphenylpropionic acid, *m*-coumaric acid, *m*-hydroxyhippuric acid, hippuric acid (see Figure 2.6 for chemical structures) and also their conjugated forms with glucuronide or sulfate. In their experiment of *in situ* absorption at the mid-ileal portion of the intestines, the results showed that 89.4% of the dose remained in the luminal fluid with more than 95% intact and only 0.2% found in mesenteric vein as intact esters, which suggests that γ -oryzanol was poorly absorbed. Additionally, it was also found that only 0.3% of the dose was transported into the thoracic duct, of which more than 80% was intact γ -oryzanol by thin layer chromatography. Therefore, it was assumed that the absorbed γ -oryzanol from oral



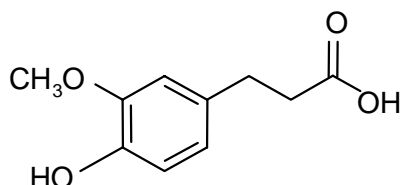
ferulic acid



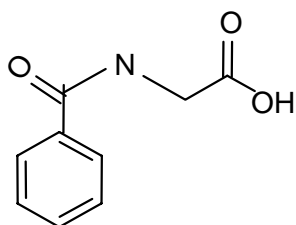
m-coumaric acid



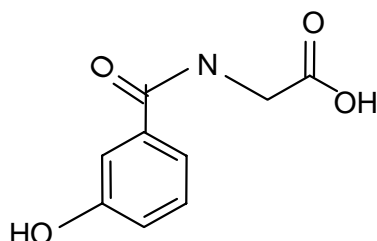
m-hydroxyphenylpropionic acid
(*m*-HPPA)



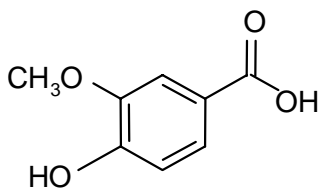
dihydroferulic acid



hippuric acid



m-hydroxyhippuric acid
(*m*-HHA)



vanillic acid

Figure 2.6. Chemical structures of ferulic acid and its urinary metabolites as reported in Fujiwara *et al.*, 1983, and Booth *et al.*, 1957

administration mainly went through the portal vein system instead of the lymph via the thoracic duct. In their study, the radioactivity found in feces was not tested as to whether it was the intact γ -oryzanol or its metabolites. It was reported that the ester linkage of γ -oryzanol was partly hydrolyzed in the intestine during absorption; however, the digestibility of γ -oryzanol going through the stomach, duodenum and jejunum was not discussed.

Even though the digestibility of γ -oryzanol has not been fully studied, absorption of plant sterols by the human body has been investigated. It was found that the plasma levels of plant sterols were very low, approximately only 5% of ingested plant sterols are absorbed, and suggested that the absorption was limited, except for those with a rare genetic disorder, sitosterolemia, which increases the absorption rate of plant sterols (Moghadasian, 2000). The accumulation of sitosterol in plasma and tissues of a sitosterolemic patient accounted for the increased sitosterol absorption rate and the decreased elimination rate and symptoms include tendon xanthomas, accelerated atherosclerosis, hemolytic episodes, arthritis and arthralgias and was observed mostly in young males.

Therefore, to fully understand the fate of γ -oryzanol in the human digestive system, further research is required. In this study, the *in vitro* digestibility of γ -oryzanol by pepsin and pancreatic enzymes such as cholesterol esterase, phospholipase A₂ and lipase was inspired and the absorbability of γ -oryzanol was demonstrated in a human intestinal cell model.

2.7 *In Vitro* Cell Culture Models

Tissue culture, commonly used as a generic term to refer to organ culture and cell culture as well as tissue culture, was first devised at the beginning of the 20th century. The term cell culture implies a culture derived from dispersed cells taken from original tissue or from a cell line. Tissue culture techniques can be applied for studies in toxicology, virology, immunology,

cancer research and so on. The advantages of tissue culture are control of physiochemical environment (pH, temperature, O₂) and physiological conditions (hormone, nutrients), production of homogeneous culture to reduce variance, avoidance of the ethical questions of animal experimentation, and economic with availability of microtitration and robotics and with direct access to the cell and consequently less use of reagent.

2.7.1 Cell Selection

To select a cell line, besides the specific function to be studied, there are a number of parameters to be considered (Freshney, 2000): 1) Finite or continuous cell line; a continuous cell line usually grows faster and is easy to maintain. 2) Normal or transformed cell line. 3) Species; non-human cell lines are more accessible and have fewer biohazard restrictions. 4) Growth characteristics such as population-doubling time, yield and cloning efficiency. 5) Availability. 6) Validation; how well the cell line is characterized as acceptable. 7) Phenotypic expression; if the cell line is made to express the right characteristics. 8) Control cell line; if a normal equivalent cell line is required when using a transformed or abnormal cell line. 9) Stability; how the cell line is stable and can be generated into sufficient frozen and usable stocks.

However, there are also limitations in developing and using *in vitro* cell models. One is the requirement of expertise due to the strict aseptic culture conditions to avoid chemical or microbial contamination and the handling of biohazard disposal. Secondly, cells may lose the phenotypic characteristics and not function as they should. Many continuous cell lines may also show variability from passage to passage. Other limitations include the loss of cell-cell interactions and homeostatic regulations from nervous and endocrine systems and the alteration of energy metabolism, which may result in cell behavior change and not be truly representative of the tissue from which the cells were isolated. Even though limitations exist, there are many

specialized functions expressed and cell culture can still be a valuable tool if the limits are appreciated.

Cell culture techniques have been used to investigate antioxidant activities and cholesterol absorption. The cell lines that have been utilized for antioxidant activity studies include rat hepatocytes (Halpner *et al.*, 1998), bovine aortic and pulmonary artery endothelial cells (Kapiotis *et al.*, 1997 and Podhaisky *et al.*, 1997), human aortic endothelial cells (Pearson *et al.*, 1998) and human umbilical vein endothelial cells (Kaneko *et al.*, 1993). The cell lines that have been used for cholesterol uptake studies include human intestinal cells, Caco-2, (Mackay *et al.*, 1997 and Lopez-Candales *et al.*, 1993) and rat intestinal cells (Young and Hui, 1999). In this study, because of its availability, growing efficiency and established utility, a mouse endothelial cell line (SVEC4-10) derived from axillary lymph node vessels was used to examine antioxidant activity of γ -oryzanol and a human intestinal cell line (C2BBel) was used to investigate the γ -oryzanol effect on cholesterol uptake.

2.7.2 Cell Viability Assays

Cell viability assays are immediate or short-term responses from cells such as a variation in membrane permeability or a disturbance of a particular metabolic pathway, usually obtained when they encounter a new environment of interest. There are several assays available, which have been described in many publications (i.e. antioxidant activity studies), including dye exclusion, crystal violet assay, lactate dehydrogenase (LDH) assay and [3-(4,5-Dimethylthiazol-yl)-2-5-Diphenyltetrazolium Bromide (MTT) assay.

Dye exclusion is a basic technique often employed in routine practice. Trypan blue is usually used and is impermeable to viable cells. However, this method does not always predict ultimate survival and tends to overestimate viability. The crystal violet assay is a fixed cell

staining. It is inexpensive and easy to use but it nonspecifically stains protein, which may not necessarily measure cell viability. The LDH assay is a method used to evaluate cell viability in terms of the degree of cell membrane integrity. An LDH assay kit can be purchased commercially. It is used to detect LDH released from surviving or dead cells. The MTT assay is a widely chosen method to estimate the number of viable cells, whose mitochondrial dehydrogenases can reduce the yellow tetrazolium dye to a purple formazan product. The selection of viability assay depends on availability and avoidance of reacting with the test substances.

2.8 Emulsion Preparation for γ -Oryzanol and Lipophilic Substances

Since γ -oryzanol is not water soluble, it has been a challenge to prepare physiological aqueous solutions containing γ -oryzanol and other hydrophobic or lipophilic substances for cell culture studies. Emulsifiers or surfactants are usually involved, which function to create surface-tension gradients and to deform the interface. They are amphipathic compounds, which contain both polar and nonpolar regions. Many biomolecules are amphipathic such as proteins, phospholipids and bile salts. When dispersed in water, the lipid portions force surrounding water molecules to become highly ordered and clusters of lipid molecules are formed. Then all hydrophobic groups are sequestered from water and form a stable structure called micelles with the hydrophobic regions inside by the force of hydrophobic interaction and the hydrophilic regions at the surface.

Micelle formation plays very important roles in digestion, mobilization and transport of lipids. Before being absorbed through the intestinal wall, dietary lipids must be converted to finely dispersed microscopic micelles with the help of amphipathic compounds, i.e. bile salts, such as taurocholate acting as biological detergents. Bile salts are synthesized from cholesterol in

the liver, stored in the gallbladder and released into the duodenum. The formation of micelles increases the action of water-soluble lipase to convert triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids and glycerol, which can diffuse into the epithelial cells lining the intestinal surface (the intestinal mucosa). Those lipase products are then reconverted to triacylglycerols and incorporated with dietary cholesterol and apolipoproteins into chylomicrons. Apolipoproteins, also amphipathic, are lipid-binding proteins in the blood responsible for the transport of triacylglycerols, phospholipids, cholesterol and cholesteryl esters between organs. Chylomicrons travel through the lymphatic system and bloodstream to tissues and form several classes of lipoproteins named very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL), according to the combinations of lipids that change constantly and cause different densities.

Therefore, phosphatidylcholine and bovine serum albumin were used to prepare γ -oryzanol emulsion to demonstrate the antioxidant effect of γ -oryzanol on protecting against oxidative stress to mouse lymphatic endothelial cells. Oleic acid, 1-monoolein, phosphatidylcholine and sodium taurocholate were used to prepare micelles containing γ -oryzanol to study γ -oryzanol digestibility and hypocholesterolemic capacity of γ -oryzanol taking place in human intestinal cells.

2.9 The Potential Functionality of γ -Oryzanol to Human Health

The concepts of food and nutrition are changing from a focus on preventing hunger and adverse effects on health and improving nutrition for health maintenance to a recognition that food may reduce morbidity and mortality from chronic diseases such as cardiovascular disease, cancers and obesity. Therefore, the science of functional foods, defined as nutrients or non-nutrients that play a role in affecting one or more targeted functions in the body in a positive way

(i.e. preventing disease) beyond the prevention or correction of nutrient deficiency, is becoming an emerging field. The term chemoprevention is sometimes used with emphasis on the function to decrease the incidence of disease by using specific chemicals, either alone or in combinations. Requirements would be that they lack significant toxicity and are easy to be supplemented for large sections of the populations.

γ -Oryzanol has been suggested to have potential functionality such as antioxidant activity (Xu and Godber, 2001), reduction of serum cholesterol (Sasaki *et al.*, 1990), reduction of cholesterol absorption and decrease of early atherosclerosis (Rong *et al.*, 1997), inhibition on platelet aggregation (Seetharamaiah *et al.*, 1990) and inhibition of tumor promotion (Yasukawa *et al.*, 1998). Hence, this study was inspired to focus on the functionality of γ -oryzanol including antioxidant activity and hypocholesterolemic capacity and the possible mechanisms of actions as determined using *in vitro* cell culture models.

2.9.1 Antioxidant Activity

In order to understand the function of antioxidants, first of all, the oxidative stress caused by the generation of reactive oxygen species (ROS) associated with the presence of oxygen even under physiological conditions and the antioxidant defense system in human organisms must be discussed. ROS are either radicals, atoms or a group of atoms that contain at least one unpaired electron, such as peroxy radicals (ROO^\bullet), hydroxyl radicals ($^\bullet\text{OH}$), the nitric oxide radical (NO^\bullet) and the superoxide anion radical ($\text{O}_2^{\bullet-}$), or non-radical compounds, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$), which are capable of oxidizing molecules. ROS may have positive, negative or zero charge and sometimes are also called oxidants or prooxidants.

The origins of ROS can be from normal human metabolism (endogenous source) starting with $\text{O}_2^{\bullet-}$, generated by enzymatic one-electron reduction of O from xanthine oxidase, NADPH

oxidase, and other reactive intermediates such as H_2O_2 and $\cdot\text{OH}^-$. The other endogenous source of ROS is generated as part of the primary immune defense by phagocytic cells such as neutrophils, monocytes or macrophages, which synthesize large amounts of $\text{O}_2^{\bullet-}$ and $\text{NO}\cdot$ against foreign organisms. Human organisms are also exposed to ROS (external sources) such as diet, cigarette smoke and ozone due to air pollution.

The antioxidant defense system *in vivo* includes enzymatic and non-enzymatic antioxidants, which are defined as any substances that in a relatively low concentration compared with substrate significantly delay or inhibit the oxidation of that substrate. The major enzymatic antioxidants are superoxide dismutase ($\text{O}_2^{\bullet-} \rightarrow \text{H}_2\text{O}_2$), catalase ($\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O}$), glutathione peroxidase (organic hydroperoxides $\text{ROOH} \rightarrow \text{H}_2\text{O}$) with glutathione ($\text{GSH} \rightarrow \text{GSSH}$), glutathione reductase to restore GSH from GSSH, glutathione-S-transferases (conjugation with some reactive intermediate products) and metal-binding proteins (ferritin and transferrin, prevention of initiation of lipid peroxidation or DNA damage).

The non-enzymatic antioxidants are dietary antioxidants among which the most prominent are ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids and flavonoids. Vitamin C acts as a scavenger of ROS, is most effective against $\text{O}_2^{\bullet-}$, H_2O_2 , $\cdot\text{OH}^-$ and $^1\text{O}_2$, and forms ascorbyl radical. Vitamin E is a scavenger of $\text{ROO}\cdot$, is more important in inhibiting lipid peroxidation, and yields tocopheroxyl radical, which is less reactive and acts as a chain-breaking antioxidant. Carotenoids are natural colorants and scavengers of $^1\text{O}_2$ and $\text{ROO}\cdot$ (physical quenching by energy transfer and chemical quenching by interaction with radicals). Flavonoids are polyphenolic antioxidants that can scavenge $\text{ROO}\cdot$, $\text{O}_2^{\bullet-}$ and $\cdot\text{OH}^-$ and form phenoxy radicals.

It has been suggested that ROS are involved in numerous diseases such as cardiovascular diseases, cancer, cataract and age-related macular degeneration, neuronal diseases and so on. For example, the main cause of many cardiovascular diseases is thought to be atherosclerosis in the artery wall, which is fatty streaks containing large cytoplasmic lipid deposits of free and esterified cholesterol formed in the subendothelial space. This occurs by way of aggregation of foam cells, which are of macrophage origin, with smooth muscle cells comprising the rest. There is increasing evidence that LDL oxidation initiated by radicals is responsible for the pathogenic pathway and lipophilic antioxidants such as α -tocopherol are very effective to inhibit LDL oxidation. Therefore, antioxidants play important roles in preventing oxidative damage to bodily functions and dietary antioxidant intake is expected to lower the risk of a number of ROS-related diseases.

Direct measurement of prooxidants *in vivo* is difficult; however, there are many studies that have established using *in vitro* models in order to speculate relative to *in vivo* antioxidant action. Generally, the models constructed to study antioxidant activity of a compound or mixture consist of three parts: 1) ROS generators to initiate oxidation reactions, i.e. 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS $^{\bullet+}$), 2,2'-azobis-2,4-dimethyl valeronitrile (AMVN), air (oxygen), UV and oxidizing agents such *tert*-butyl hydroperoxide (tBHP) and H₂O₂. 2) Substrates that are the targets of ROS, i.e. cholesterol, fatty acids such as linoleic acid, fluorescent probe such as fluorescein, LDL and cells cultured *in vitro*. The degree of oxidative damage to the substrates prevented by the test compound is usually determined directly or indirectly through the decrease of substrates (i.e. fluorescence decay and cell viability) or the increase of oxidized products (i.e. oxidized cholesterol, oxidized linoleic acid, lipid peroxidation intermediates such as

thiobarbituric acid reactive substances (TBA-RS) and conjugated dienes). 3) detection methods to allow monitoring the action such as HPLC for lipid hydroperoxides, spectrometer, spectrofluorometer and cell viability assays.

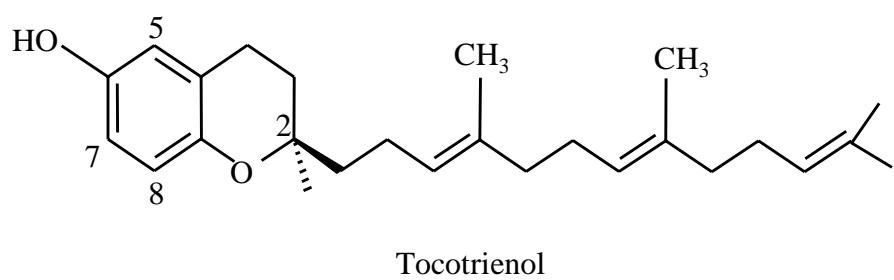
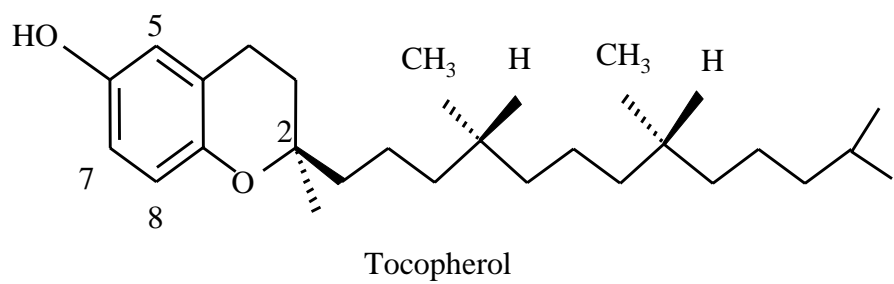
The antioxidant activity of γ -oryzanol has been evaluated in a few models using chemicals to induce oxidation and measuring the generation of oxidized products. One of them is a cholesterol oxidation model accelerated by AAPH, which was established to compare the inhibitory capability of γ -oryzanol and vitamin E on production of oxidized cholesterol (Xu and Godber, 2001). The antioxidant activity was determined in terms of the degree to which the generation of seven oxidized cholesterol products (7- α - and - β -hydroperoxycholesterol, 5,6- α - and - β -epoxycholesterol, 7- α - and - β -hydroxycholesterol and 7-ketocholesterol) was prevented in the presence of three major components of γ -oryzanol (cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate) or vitamin E components (α -tocopherol, γ -tocopherol, α -tocotrienol, γ -tocotrienol). The results suggested that 24-methylene cycloartanyl ferulate had the highest antioxidant activity and all three major components of γ -oryzanol had higher antioxidant activity than vitamin E components.

Another assessment of antioxidant activity of γ -oryzanol using AAPH as a peroxyl radical generator utilized an oxygen radical absorbance capacity assay (Huang *et al.*, 2002). This assay used cyclic (α -1,4)-linked oligosaccharides of α -D-glucopyranose, which have both hydrophilic and hydrophobic regions, to prepare lipophilic antioxidants such as vitamin E isomers and γ -oryzanol, and fluorescein as a target of free radical attack. Antioxidant activity was determined as the degree to which the fluorescence decay of fluorescein caused by AAPH was inhibited over time. Similarly, their results showed that γ -oryzanol possessed the greatest antioxidant activity compared to other antioxidants tested including α -tocopherol, γ -tocopherol,

δ -tocopherol and tocotrienols, while α -tocopherol acetate had zero effect. It seemed that γ -oryzanol had the greatest antioxidant activity towards peroxy radicals generated by AAPH and it was proposed that the antioxidant mechanism of γ -oryzanol might be due to its intramolecular hydrogen-bonded methoxyphenols, where hydrogen donation would occur more readily.

It has also been postulated that the phenol group is the key functional group for antioxidant activity and that steric hindrance around the phenol group decreases antioxidant activity. For example, in their model of the antioxidant activity of tocopherols, Huang *et al.* (2002) found that relative order of antioxidant activity was $\alpha < \gamma < \delta$ and the reason was explained relative to the number of methyl groups *ortho* to the phenol group where α has two, γ has one and δ has zero (see Figure 2.7 for vitamin E structure). The theory has also been approached in a review of the antioxidant chemistry of ferulic acid (Graf, 1992). It was explained that the antioxidant activity of ferulic acid is due to its phenolic hydroxyl group that has hydrogen donating property to scavenge a reactive radical and form a phenoxy radical. This radical is then stabilized resonantly because the unpaired electron can be delocalized across the entire molecule (Figure 2.8). The second phenolic hydroxyl group can enhance the radical-scavenging property by providing additional resonance stabilization and form quinone. The methoxyl group of ferulic acid partially destabilizes the phenoxy radical and impairs its antioxidant activity.

However, in a study using $\text{ABTS}^{\bullet+}$ as a radical generator to study the antioxidant activities of benzoic acid or cinnamic acid as a parent acid and other acids (i.e. ferulic acid) with hydroxyl groups and methoxyl groups at different positions, the results conflicted with the proposed antioxidant mechanism (Miller and Rice-Evans, 1997). Their results showed that vanillic acid (3-methoxy-4-hydroxy benzoic acid) had a higher antioxidant activity than 4-hydroxy benzoic acid (Figure 2.9 for chemical structures). Two hydroxyl groups attached to



Position of methyl group	Tocopherols	Tocotrienols
5,7,8-Trimethyl	α -T	α -T3
5,8-Dimethyl	β -T	β -T3
7,8- Dimethyl	γ -T	γ -T3
8-Monomethyl	δ -T	δ -T3

Figure 2.7. Chemical structures of tocopherols and tocotrienols

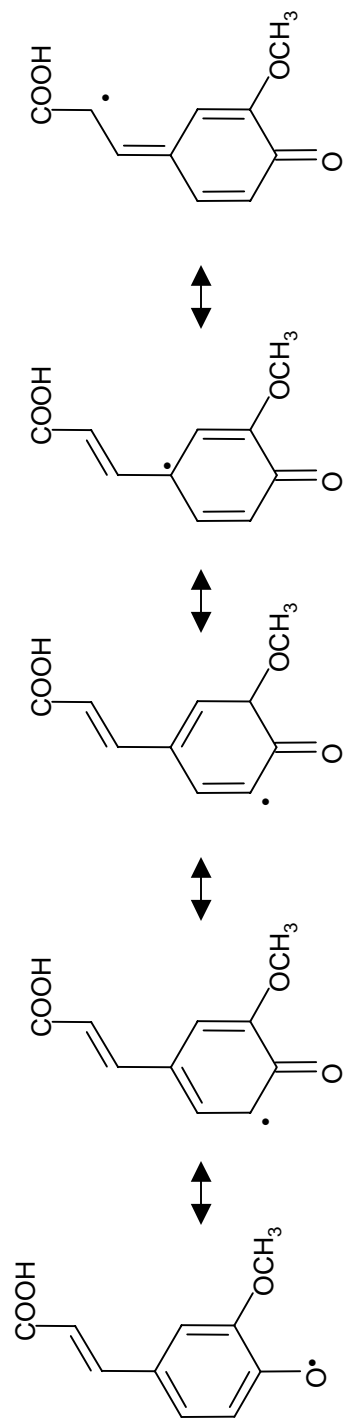
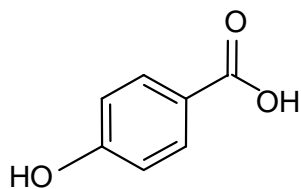
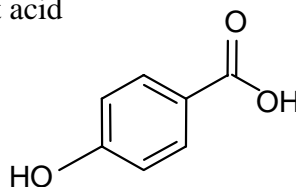
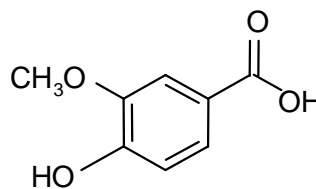


Figure 2.8. Resonance stabilization of ferulic acid radical from Graf, 1992

(a) benzoic acid as parent acid

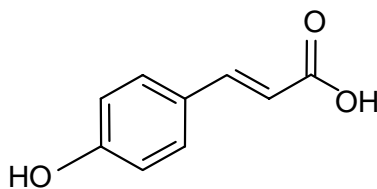
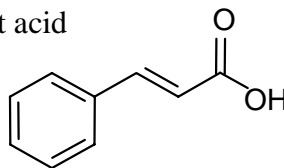


p-hydroxy benzoic acid

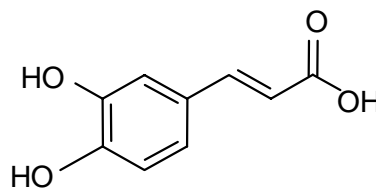


vanillic acid

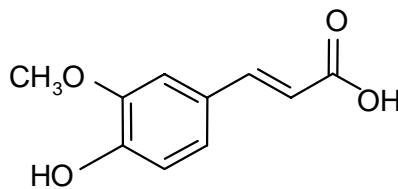
(b) cinnamic acid as parent acid



p-coumaric acid



caffeic acid



ferulic acid

Figure 2.9. Chemical structures of (a) hydroxybenzoic acids and (b) hydroxycinnamic acids

benzoic acid had higher antioxidant activity than one hydroxyl group but did not necessarily have higher antioxidant activity than vanillic acid. On the other hand, ferulic acid (3-methoxy-4-hydroxy cinnamic acid) did not have higher antioxidant activity than 4-hydroxy cinnamic acid but had higher antioxidant activity than caffeic acid (3,4-dihydroxy cinnamic acid). Therefore, it is difficult, with the available evidence, to postulate the relationship between antioxidant activity and structure and to explain whether or not ferulic acid esterified with sterols or triterpenes (γ -oryzanol) would have a higher antioxidant activity.

Antioxidant activity of the three major components of γ -oryzanol was also approached in a linoleic acid model (Xu and Godber, 2001). Antioxidant activity was determined as the degree of inhibition on hydroperoxide formation that resulted from linoleic acid incubated with air flow at 37°C for 40 to 200 minutes. There were four hydroperoxide isomers, 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid [9HPODE(*t,c*)], [9HPODE(*t,t*)], [13HPODE(*c,t*)] and [13HPODE(*t,t*)] measured by normal-phase HPLC and UV detection. The results showed that γ -oryzanol incubated with linoleic acid at molar ratios of 1:100 and 1:250 significantly decreased the formation of hydroperoxides, especially *trans,cis* and *cis,trans* forms, which was similar to the antioxidant activity of ferulic acid. α -Tocopherol was also tested and showed significant antioxidant activity but it was more effective in preventing the formation of *trans,trans* hydroperoxides, which may indicate a different antioxidant mechanism from that of γ -oryzanol or ferulic acid. It has been reported that the hydroxyl group in the phenolic ring of ferulic acid may be responsible for the antioxidant activity. In this case, ferulic acid presented a higher antioxidant activity than γ -oryzanol, especially at a lower concentration. While γ -oryzanol also has ferulic acid in its structure, it may suggest that the sterol or triterpene alcohol portion of the

γ -oryzanol structure may affect the antioxidant activity by reducing the mobility because of the much larger molecular structure.

In spite of the efforts contributed by these researchers in studies of γ -oryzanol antioxidant activity, the functionality of γ -oryzanol as an antioxidant in the human body has not been delineated. One of the objectives of this study, therefore, was to develop a cell culture model *in vitro* as a closer approach to the human body and use it to compare the antioxidant activity of γ -oryzanol and its three major components to α -tocopherol. The cell line used to develop the cell culture model was SVEC4-10, a mouse endothelial cell line derived by SV40 (strain 4A) transformation of endothelial cells from axillary lymph node vessels. The oxidizing agent was *tert*-hydroperoxide. The MTT, [3-(4,5-dimethylthiazol-yl)-2-5-diphenyltetrazolium bromide, assay was chosen to measure cellular mitochondrial activity as an index of cell viability. Lipid peroxidation was also analyzed to detect TBA-RS and conjugated diene formation. Thus, the antioxidant activity of γ -oryzanol was determined as the degree to which γ -oryzanol preserved cellular mitochondrial activity and prevented lipid peroxidation from tBHP oxidative damage.

2.9.2 Hypocholesterolemic Capacity

A hypocholesterolemic effect of different vegetable oils blended with rice bran oil was found in healthy young women fed for 7 days (Suzuki and Osima, 1970). This effect was also reported in rats fed a normal diet containing 7% rice bran oil or a high cholesterol diet containing 1% cholesterol and 7% rice bran oil for 4-7 weeks and there was also significantly decreased levels of serum and liver total cholesterol and LDL and a slightly increased level of HDL in both the normal diet and the high cholesterol diet (Sunitha *et al.*, 1997).

It was also discovered that the unsaponifiable fractions in rice bran oil had cholesterol-lowering potential (Kiribuchi *et al.*, 1983, and Ikeda *et al.*, 1985). It was found that rats fed a

cholesterol-enriched (0.5%) diet with 0.05-1% of cycloartenol, 24-methylene cycloartanol and/or β -sitosterol greatly reduced plasma cholesterol and enhanced cholesterol fecal excretion.

Later, γ -oryzanol was found to be responsible for the effect (Seetharamaiah and Chandrasekhara, 1989). The rice bran oil (10%) diet showed markedly lower serum and liver total cholesterol levels in rats and rice bran oil spiked with γ -oryzanol (0.5%) reduced it even more significantly. This was also confirmed by Rong *et al.* (1997) in hamsters fed a diet containing 0.1% cholesterol with or without 1% γ -oryzanol for 7 weeks. The γ -oryzanol-fed animals had 28% reduction in plasma total cholesterol and 34% reduction of combined LDL and VLDL, as well as 25% decrease in cholesterol absorption. Moreover, γ -oryzanol showed no effect on endogenous cholesterol synthesis, as measured by the liver and intestinal HMG-CoA reductase activities, which is an important indication that the decreased cholesterol level in plasma may have resulted from lower cholesterol absorption and that cholesterol synthesis by HMG-CoA reductase may not increase to adjust the cholesterol level in response to the decreased cholesterol uptake.

There are only a few γ -oryzanol studies on humans. Sasaki *et al.* (1990) studied the effects of γ -oryzanol on serum lipids of twenty chronic schizophrenic patients with dyslipidemia. Each patient was given 100mg of γ -oryzanol three times daily for 16 weeks. The results showed that total cholesterol and LDL levels decreased significantly with no side effects recorded, suggesting that γ -oryzanol is safe and effective in the treatment of dyslipidemia.

Since hypercholesterolemia is often a risk factor for many heart diseases associated with platelet aggregation and atherosclerosis, the cholesterol-lowering potential of γ -oryzanol may also play important roles in preventing these diseases. The γ -oryzanol effect to reduce early atherosclerosis was also observed in hamsters fed a diet containing 0.05% cholesterol and 10%

coconut oil with or without 0.5% γ -oryzanol (Rong *et al.*, 1997). The results showed that the aortic fatty streak formation, as determined by the degree of accumulation of macrophage-derived foam cells, was reduced 67%. In addition, the influence of γ -oryzanol on platelet aggregation induced by ADP and collagen was also reported in rats fed a 1% cholesterol diet containing γ -oryzanol (Seetharamaiah *et al.*, 1990). The platelet aggregation in platelet rich plasma induced by both ADP and collagen was significantly inhibited in γ -oryzanol-treated rats.

In summary, γ -oryzanol has shown promising hypocholesterolemic capacity. To postulate the hypocholesterolemic mechanism of γ -oryzanol, the cholesterol absorption and transport in humans must be discussed. The cholesterol in the human body comes from two sources. One is endogenous cholesterol, which is synthesized in the liver. Some may go into VLDL and be secreted into the blood and some are converted to bile salts, stored in the gallbladder, released into the duodenum as biological detergents to form micelles with dietary lipids, reabsorbed at the ileum and return to the liver through the hepatic portal vein. The other source is exogenous dietary cholesterol, which is incorporated into micelles, absorbed at the jejunum and travels to the liver through the lymphatic system and bloodstream. During any one day, the cholesterol in the intestinal lumen is typically two-thirds from endogenous sources and one-third from dietary sources. Cholesterol absorption in humans can vary widely (15 to 75%) (Grundy, 1983). It has been demonstrated that decreased serum cholesterol, specifically LDL, can result from the inhibition of cholesterol absorption (Gylling and Miettinen, 1995). According to this mechanism, drugs such as questran and cholestyramine, which are powdered resins that increase excretion of bile salts have been prescribed by doctors for hypercholesterolemic patients.

Due to the rising controversial issue about using laboratory animals, cell culture models have been used for many cholesterol uptake and cholesterol synthesis research projects. Since γ -

oryzanol digestibility is unclear, whether or not γ -oryzanol can be absorbed and function in the human body i.e. inhibition of cholesterol synthesis in liver is beyond the scope of this study. This study focused on the γ -oryzanol effects on the availability of cholesterol for uptake into *in vitro* human intestinal cells, especially the effects on micellar solubility of cholesterol (free and ester forms) and on inhibition of cholesterol esterase to hydrolyze cholesteryl esters.

2.10 Applications of γ -Oryzanol

Since the functionality of γ -oryzanol has been found to be promising, rice bran or γ -oryzanol may have great market potential and be applied to a wide range of products. It can be enriched in food products such as cereal and margarine for its cholesterol-lowering and antioxidant effects, put into frying oil for its stability and into food coating or packaging material for antioxidant potential to extend shelf life. It can also be supplemented into non-food products such as sun protection skin lotion due to UV absorption and skin-care products for repairing dry and sensitive skin.

CHAPTER 3. ANTIOXIDANT ACTIVITY EXPERIMENTS USING AN *IN VITRO* MOUSE LYMPH AXILLARY ENDOTHELIAL CELL MODEL

3.1 Introduction

The potential antioxidant activity of γ -oryzanol has been studied in chemical models such as in a cholesterol model to reduce cholesterol oxidation accelerated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (Xu *et al.*, 2001) and in a linoleic acid model to prevent linoleic acid oxidation induced by air (Xu and Godber, 2001). The antioxidant activity of ferulic acid, part of γ -oryzanol structure, has also been investigated as determined by its ability to scavenge ABTS^{•+} radical cation more effectively than vitamin C (Miller and Rice-Evans, 1997) and to inhibit lipid peroxidation (malondialdehyde production) in rat brain homogenates *in vitro* (Sharma, 1976). However, the functionality of γ -oryzanol as an antioxidant in the human body has not been delineated. The objectives of this study, therefore, were to develop an *in vitro* cell model assay as an approach that more closely reflects what might occur in the human body and use it to evaluate the effect of γ -oryzanol. The latter could be distinguished by testing its ability to preserve cellular mitochondrial activity from damage caused by *tert*-hydroperoxide (tBHP) compared with the effect of α -tocopherol. The antioxidant activity of the three major components of γ -oryzanol and the possible synergism among γ -oryzanol, ferulic acid and α -tocopherol were also studied.

3.2 Materials and Methods

3.2.1 Materials and Reagents

Milled rice bran was obtained from Riviana Rice Mill, Abbeville, LA. Distilled and deionized water was used to prepare culture media and solutions. Ferulic acid, α -tocopherol, lecithin, fatty acid free bovine serum albumin, MTT assay kit, LDH test kit, Bradford reagent,

Hank's balanced salts, thiobarbituric acid, butylated hydroxytoluene and trypan blue solution were purchased from Sigma Chemical Co., St. Louis, MO. Sodium bicarbonate and HCl were purchased from Mallinckrodt, Inc., Paris, KY. All the solvents used were HPLC grade. Methanol, hexane, dichloromethane, acetonitrile, acetic acid and trichloroacetic acid were purchased from Fisher Scientific, Fair Lawn, NJ. Ethanol was USP absolute 200 proof purchased from AAPER Alcohol and Chemical Co., Shelbyville, KY. Culture plates, flasks and scrapers were purchased from Corning Inc., Corning, NY, and the sterile disposable syringe filter was 0.2 μ m PES purchased from Whatman, Clifton, NJ.

3.2.2 Extraction of γ -Oryzanol from Rice Bran and Chemical Analysis of γ -Oryzanol, Ferulic Acid and α -Tocopherol

Solvent extraction of rice bran was done to obtain crude rice bran oil. Two hundred grams of milled rice bran were mixed with hexane and then filtered and the hexane extraction was repeated for a total of 3 times. The hexane layer was combined into a round-bottom bottle, followed by vacuum evaporation (45°C, CentriVap Console, Labconco Inc., Kansas City, MO) to remove hexane and yield crude oil. To separate γ -oryzanol from the oil, a preparative normal-phase high performance liquid chromatography (HPLC) procedure developed by Xu and Godber (1999) was used. The preparative HPLC system consisted of a Waters (Milford, MA) Prepak[®] RCM base connected with two cartridges (each 25mm \times 10cm Prep Nova-Pak HR silica with particle size 6 μ m) and a Guard-Pak insert, a U6K manual injector, a 510 pump and a Lambda-Max 481 LC Spectrophotometer (wavelength 330 nm). Mobile phase was methanol with a constant flow rate at 15 mL/min and 2mL of the crude oil were injected each time. The eluate containing γ -oryzanol was collected (25-30min) and the solvent was evaporated using a vacuum evaporator.

To separate γ -oryzanol fractions, the HPLC system used consisted of a pump (Waters 510 HPLC Pump), an autosampler (Waters 715 Sample Processor), a column (C_{18} , MICROSORB-MV 100 Å, Varian) and a UV detector (Waters 486 Tunable Absorbance Detector) at wavelength 330 nm. The mobile phase was methanol, acetonitrile, dichloromethane and acetic acid (50:44:3:3) and the flow rate was 1.4 mL/min. The γ -oryzanol obtained from rice bran oil was dissolved in the mobile phase. Ultrasonication was used to help dissolve when necessary. One hundred μ L of γ -oryzanol solution (1.5mg/mL) was injected each time. Cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate were collected (at retention time 5.2-5.8, 5.8-6.3 and 6.3-6.8 minutes, respectively), dried using a vacuum evaporator, sealed with foil and kept frozen until further experiments.

Determination of γ -oryzanol, ferulic acid and α -tocopherol concentrations was obtained from retention time comparison and quantifications of known standards. The HPLC system to analyze α -tocopherol was the same as for γ -oryzanol except that the detector was a Waters 470 Scanning Fluorescence Detector with excitation wavelength set at 290nm and emission at 330nm.

3.2.3 Emulsion Preparation

Because γ -oryzanol and α -tocopherol are hydrophobic, emulsifiers such as lecithin and bovine serum albumin (BSA) were needed to prepare γ -oryzanol and α -tocopherol in aqueous culture solutions. γ -Oryzanol, α -tocopherol or ferulic acid was dissolved in ethanol (50mM) and filtered through 0.2 μ m filter for sterilization and the concentration was checked by HPLC. Each was then diluted to make 2mM stock solution in filtered Hank's balanced salt solution (HBSS)-emulsion composed of lecithin and essential fatty acid free BSA, whose concentrations were

tested first to assess impact on cell viability. The emulsion was then evaluated to be free of precipitation. For cell culture experiments, the final ethanol concentration did not exceed 2%.

3.2.4 Cell Culture

For antioxidant activity experiments, mouse lymph axillary endothelial cell line, SVEC4-10 (ATCC CRL-2181), was chosen. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4mM L-glutamine, 4.5g/L glucose, 1.5g/L sodium bicarbonate, 15mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 10% fetal bovine serum in a 37°C 5% CO₂ humidified incubator. To maintain the cell line, cells were detached from surface using 0.25% trypsin solution and split to new 25-cm² culture flasks (1:5-1:7 split ratio). Cells reached confluency to form a monolayer in about 3-4 days.

Cells for antioxidation experiments were cultured in 96-well (12x8) cell culture plates. After subculture as described above, cell suspension was diluted in new medium to at least 10⁵ cells/mL and 100μL of the diluted cell suspension was transferred to each well. If not all 96 wells were used, only the middle wells of the plate would be used to prevent "edging effect" due to uneven evaporation and the unused wells were filled with water to maintain humidity. An exact cell counting was performed at this time (as described below). The plates were kept at 37°C in the humid incubator until they reached confluency (about 3-4 days) for further experiments.

3.2.4.1 Growth Curve

After obtaining a cell line, it is recommended to construct a growth curve in order to help understand the cell growth characteristics. Cell counting using dye such as trypan blue and the hemacytometer is one of the commonly used methods. For adhesive cell lines including the one used in this project, after trypsinization, 200μL of cell suspension to be counted was mixed with

200µL 0.4% trypan blue solution. Pipetting up and down at least 20 times was done to break the cell clumps. A drop of the cell suspension was placed to the edge of one side of the hemacytometer covered with the coverslip and repeated for the other side. The cell suspension was drawn by the force of capillary action. The hemacytometer was then transferred to the microscope with a standard 10x objective. The counting chamber of the hemacytometer consisted of nine squares and each square had the volume of 10^{-4} mL (1-mm long \times 1-mm wide \times 0.1mm deep). For cell viability, only the “sort of healthy” to viable cells, which were impermeable to trypan blue and appeared clear, were counted, as opposed to sick cells, which could not exclude dye and appeared dark. At least 100 cells per square were counted. Cell count was calculated as viable cell numbers per mL, which was the average cell number counted in one square times 10^4 and times 2 with 1:1 trypan dilution. Cell counting was done everyday for 6 days after subculture. The SVEC4-10 cell growth curve from subculture was expressed as cell count in logarithmic scale versus days.

3.2.4.2 MTT and LDH Assays for Cell Viability

To measure cell viability under oxidative stress, an MTT assay kit was used to measure the activity of mitochondrial dehydrogenases in living cells. After the test solution was removed and the cells were washed three times with HBSS, 40µL of 1.25mg/mL MTT in HBSS, which is yellow color, was added to cells. After incubation at 37°C in the dark for 2 hours, the mitochondrial dehydrogenases of viable cells cleaved the tetrazolium ring and yielded purple formazan crystals, which are insoluble in aqueous solution. Then 100µL/well of MTT solubilization solution containing 10% Triton X-100 plus 0.1N HCl in anhydrous isopropanol was added to solubilize purple formazan crystals. Pipetting up and down at least 100 times helped solubilization in dense culture situation. Absorbances at 570nm for color development

and at 690nm for background were read using a microplate spectrophotometer (SPECTRAmax® PLUS, Molecular Devices Co., Sunnyvale, CA). An increase or decrease of cell numbers after exposure to a test solution would correspond to a concomitant change in the amount of purple formazan formed or in the optical density at absorbance 570nm. The cell viability under exposure to a test compound was expressed in %cellular mitochondrial activity, which was the ratio of absorbance reading difference between 570nm and 690nm of test compound ($A_{570} - A_{690}$) to that of control ($A_{570}^0 - A_{690}^0$):

$$\% \text{Cellular mitochondrial activity} = \frac{A_{570} - A_{690}}{A_{570}^0 - A_{690}^0} \times 100\%$$

The lactate dehydrogenase (LDH) assay was another method used to evaluate cell viability in terms of the degree of cell membrane integrity. It was used to detect LDH released from both surviving and dead cells. To measure LDH released from damaged cells, 50µL/well of the cell supernatant to be measured was transferred to a new plate and mixed with 25µL/well of a freshly prepared LDH assay solution containing dye, along with substrate and enzyme (1:1:1). To measure LDH in surviving cells, the cells were washed with HBSS three times to remove test solution and a cell lysis solution (1% sodium dodecyl sulfate (SDS) in culture grade water) at 100µL/well was applied to cells for 45 minutes, followed by 50µL/well LDH assay mixture as described above. After incubation at room temperature in the dark for 30 minutes, absorbances at 490nm for color development and at 690nm for background were read using a spectrophotometric plate reader.

3.2.5 Cytotoxicity Studies of γ -Oryzanol, Ferulic Acid and α -Tocopherol Emulsion

Since the compounds of interest were prepared in ethanol and diluted in HBSS-emulsion containing emulsifiers, lecithin and BSA, each one was tested for cytotoxicity. After incubation

in any situation, the test emulsions were removed and discarded and cells were gently washed with HBSS 3 times. MTT tests were then performed to monitor cell viability. The highest concentrations of each test compound without cytotoxicity would be used to study its antioxidant activity.

3.2.5.1 Cytotoxicity Studies of Emulsifiers, Lecithin and BSA

First, the cytotoxicity of emulsifiers such as lecithin and BSA that were needed to dissolve hydrophobic compounds in aqueous solution was studied. To determine the concentration of lecithin and BSA that could be used to prepare emulsion without affecting cell viability, 50 μ L/well of each lecithin \times BSA combination was added to confluent cells and followed by 50 μ L/well HBSS. Final concentration of lecithin at 0, 0.05, 0.10, 0.15, 0.20 and 0.25mM and essential fatty acid free BSA at 0, 1, 3, 5, 7 and 9mg/mL were prepared in HBSS in a 6x6 factorial design. The cells were incubated at 37°C with a humidifier tray filled with water for 4.5 hours, followed by cell washing and MTT assay for cell viability compared to the control (HBSS).

3.2.5.2 Cytotoxicity Studies of 2% Ethanol in Lecithin \times BSA HBSS-Emulsion

Since ethanol was required to dissolve γ -oryzanol or α -tocopherol, the cytotoxicity of 2% ethanol, which was the final concentration after diluting in HBSS-emulsion containing lecithin and BSA, was also considered. After SVEC4-10 cells reached confluency, 100 μ L of 2% ethanol in HBSS-emulsion, containing 0.25mM lecithin and 5mg/mL BSA determined previously, was incubated with cells at 37°C for 4 or 22 hours, followed by cell washing and MTT assay, along with HBSS or HBSS-emulsion without ethanol for comparison.

3.2.5.3 Cytotoxicity Studies of γ -Oryzanol, Ferulic Acid and α -Tocopherol

The purpose was to determine the maximum concentration of γ -oryzanol, ferulic acid and α -tocopherol without affecting cell viability prior to the antioxidant activity studies. γ -Oryzanol, α -tocopherol or ferulic acid emulsion (2% ethanol, 0.25mM lecithin and 5mg/mL BSA in HBSS) in a serial array of concentrations up to 1.0mM was added to confluent SVEC4-10 cells (100 μ L/well) for 4 or 23 hours at 37°C. The cytotoxicity of three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate was also tested. Each one was incubated with confluent cells for 1 hour at concentrations up to 1mM and for 22 hours at concentrations up to 0.50mM.

3.2.5.4 Cytotoxicity Studies of Interactions among γ -Oryzanol, Ferulic Acid and α -Tocopherol

For possible synergistic effects among γ -oryzanol, ferulic acid and α -tocopherol, an additional set of cytotoxicity experiments for combinations was also tested. Each combination was incubated with cells (100 μ L/well) at concentrations of 0.5mM γ -oryzanol+0.5mM α -tocopherol, 0.5mM γ -oryzanol+0.3mM ferulic acid, 0.5mM α -tocopherol+0.3mM ferulic acid or 0.25mM γ -oryzanol+0.25mM α -tocopherol+0.2mM ferulic acid for 1 hour at 37°C or at total concentrations up to 0.50mM of γ -oryzanol+ α -tocopherol, γ -oryzanol+ferulic acid, α -tocopherol+ferulic acid or γ -oryzanol+ α -tocopherol+ferulic acid for 22 hours.

3.2.6 Oxidative Stress by *tert*-Butyl Hydroperoxide

The oxidation agent tBHP was used to cause oxidative stress in cells and the tBHP oxidation conditions for antioxidant activity experiments depended on how long the test emulsion was incubated with cells. Without preincubation with test solution for 22 hours, cells were simultaneously incubated with 50 μ L/well of HBSS-emulsion that would be used to prepare

each test compound (4% ethanol, 0.50mM lecithin and 10mg/mL BSA in HBSS) and 50 μ L/well of tBHP in a final concentration of 0, 8, 12, 16 or 20mM in HBSS for 0, 15, 30, 45 or 60 minutes at 37°C.

If preincubated with 100 μ L/well of 2% ethanol HBSS-emulsion, which would contain each test compound later for antioxidant activity studies, for 22 hours at 37°C, after cell washing with HBSS 3 times, cells would then be incubated with tBHP at concentrations of 0, 4, 8 or 12mM in HBSS for 0, 15, 30, 45, 60 minutes at 37°C. After the oxidation time in both conditions, the cells were gently washed with HBSS 3 times and placed in HBSS until the longest incubation time was concluded and ready for MTT tests, i.e. 0 oxidation time means tBHP was added and removed immediately, washed and replaced by HBSS for 60 minutes and 15 minutes of oxidation means 15 minutes in tBHP plus 45 minutes in HBSS and so on. The tBHP concentration that could cause significant cell death would be used for antioxidant activity studies.

3.2.7 Studies of Antioxidant Activity and Synergism of γ -Oryzanol, Ferulic Acid and α -Tocopherol

Antioxidant activity, in terms of the degree of tBHP oxidative damage on mitochondria in SVEC4-10 cells that any test compound would reduce, was expressed as %cellular mitochondrial activity (the percentage of tBHP/HBSS of each test solution at a certain oxidation time compared to that at 0 oxidation time or at a certain concentration compared to the control emulsion). All experiments were conducted on confluent cells and after oxidation in any conditions, the cells would be gently washed with HBSS three times, followed by the MTT assay to monitor cell viability. The antioxidant activity of γ -oryzanol and ferulic acid, compared to α -tocopherol, along with the three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate, and the synergistic effect among γ -oryzanol,

ferulic acid and α -tocopherol were studied. The interferences of γ -oryzanol, α -tocopherol or ferulic acid with tBHP or MTT were also tested.

3.2.7.1 Oxidation Time Effect on Antioxidant Activity of γ -Oryzanol, Ferulic Acid and α -Tocopherol

There were four conditions designed to approach the antioxidant activity. For simultaneous presence of the test compound and tBHP, cells were incubated with 50 μ L/well of each test solution (2.0mM γ -oryzanol or α -tocopherol or 1.2mM ferulic acid) and 50 μ L/well of HBSS or 20mM tBHP for 0, 15, 30, 45 or 60 minutes. For pretreatment of test compound with cells before tBHP oxidation, after being preincubated with 100 μ L/well of each test solution (0.50mM γ -oryzanol, α -tocopherol or ferulic acid) for 22 hours at 37°C, cells were 1) washed with HBSS three times and followed with 100 μ L/well of HBSS or 12mM tBHP for 0-60 minutes, or 2) not washed, continuously accompanied by each test solution, and 20 μ L/well of HBSS or 72mM tBHP (resulting in 12mM in the 120 μ L/well of mixture) immediately added and incubated for 0-60 minutes. For the effect of each test compound on tBHP oxidation before introduction to cells, 2.0mM γ -oryzanol or α -tocopherol or 1.2mM ferulic acid was incubated with HBSS or 40mM tBHP (1:1) in a 4-mL vial for 22 hours at 37°C and then 100 μ L of each mixture was added to cells for 0-60 minutes.

3.2.7.2 Antioxidant Activity of The Three Major Components of γ -Oryzanol

The antioxidant activity of the three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate, at different concentrations was investigated. After reaching confluency, SVEC4-10 cells were preincubated with 100 μ L/well of each test solution up to 0.50mM for 22 hours at 37°C, followed by gently washing and 100 μ L/well of HBSS or 12mM tBHP for 1 hour at 37°C.

3.2.7.3 Synergistic Antioxidant Activity of γ -Oryzanol, Ferulic Acid and α -Tocopherol

For simultaneous presence of each test compound with tBHP, cells were incubated with 50 μ L/well of each combination of test solution (1.0mM γ -oryzanol+1.0mM α -tocopherol, 1.0mM γ -oryzanol+0.6mM ferulic acid, 1.0mM α -tocopherol+0.6mM ferulic acid or 0.6mM γ -oryzanol+0.6mM α -tocopherol+0.4mM ferulic acid) and 50 μ L/well of HBSS or 20mM tBHP for 60 minutes. For pretreatment with the test compound, cells were preincubated with 100 μ L/well of each test solution (0.25mM γ -oryzanol+0.25mM α -tocopherol, 0.25mM γ -oryzanol+0.25mM ferulic acid, 0.25mM α -tocopherol+0.25mM ferulic acid or 0.20mM γ -oryzanol+0.20mM α -tocopherol+0.20mM ferulic acid) for 22 hours at 37°C, followed with cell washing and 100 μ L/well of HBSS or 12mM tBHP for 60 minutes.

3.2.8 Lipid Peroxidation and Bradford Protein Assays

To measure the antioxidant activity against lipid peroxidation, two commonly used methods, thiobarbituric acid-reactive substances (TBA-RS) method and conjugated diene formation were performed. The TBA-RS method is to measure one of the oxidation intermediate products, malonaldehyde, which will react with TBA and gives pink color, as an indicator of lipid peroxidation. A modified method from McDonald and Hultin (1987) was used. TBA-RS reagent was prepared prior to use: TBA (final concentration 0.375%) was first dissolved in 0.25N HCl with heating and stirring if necessary and then trichloroacetic acid (final concentration 15%) was added. SVEC4-10 cells for TBA-RS assay were cultured in 25-cm² culture flasks. After reaching confluency, cells were gently washed three times with HBSS and followed with preincubation in 8mL of 0.5mM γ -oryzanol, α -tocopherol, ferulic acid, their combinations or each of the three major components of γ -oryzanol at 37°C for 22 hours. Each test solution was performed independently in a culture flask. After preincubation, the test

solution was removed, and the cells were gently washed three times with HBSS and then treated with 8mL of HBSS or 12mM tBHP for 1 hour at 37°C. After incubation, HBSS or tBHP solution was removed and the cells were gently washed with HBSS three times and scraped off into 1mL of cold deionized water. Each cell suspension was transferred to a 4-mL vial and ultrasonicated (Sonic Dismembrator, Fisher Scientific, Fair Lawn, NJ) at output 3 watts for 1 minute to homogenize. Each test sample (750µL) or standard 1,1,3,3-tetramethoxypropane (TMP) (0, 0.05 and 0.10 µg/mL) was transferred to a 15-mL test tube with cap and mixed with 1.5mL of TBA-RS reagent and 50µL of 2% butylated hydroxytoluene in ethanol. The tubes were boiled for 15 minutes, cooled in running water for 10 minutes and centrifuged at 2000g for 40 minutes. The supernatant was then transferred to acryl cuvettes to read absorbance. First, the absorption spectrum was scanned from 350 to 800nm. The wavelength with the highest absorbance was adjusted for background by subtracting the wavelength with lowest absorbance, which was then used to determine concentration of TBA reactive substances in each test sample. A standard curve was generated using TMP. The crude protein concentration in remaining cell suspension was tested by a Bradford protein assay. The same experiment of TBA-RS and Bradford protein assays was repeated again on a different day.

The excess TBA reactive substances produced in each sample during oxidation were expressed in µg of TMP equivalent per gram of protein in tBHP minus that in HBSS. Antioxidant activity of each test solution in terms of the degree of excess TBA reactive substances prevented was compared to the control. The lower the excess TBA reactive substances generated in a test solution during oxidation, the higher the antioxidant capacity.

Conjugated diene formation is another index for lipid peroxidation. A method modified from Toschi *et al* (2000) was used. After cells were subcultured in 25-cm² flasks and had

reached confluency, they were preincubated with 2% ethanol in HBSS-emulsion at 37°C for 22 hours. The emulsion was then removed and cells were washed with HBSS three times and treated with 8mL of HBSS or 12mL tBHP for 1 hour. Cells were again gently washed three times with HBSS, scraped off into 1mL of methanol and extracted in chloroform/methanol/H₂O (2:1:1) three times. The chloroform layers were collected and combined and evaporated under nitrogen gas. The residue of each sample was then dissolved in 500μL of acetonitrile and the absorbance spectrum from 190 to 250nm was scanned.

For Bradford assay to measure crude cell protein, two aliquots of 5 to 10μL homogenized cell suspension were made into 1/100 to 1/200 dilutions with deionized water and mixed with Bradford reagent (1:1) for 5 minutes at room temperature in the dark. Similarly, the spectrum from 350 to 800nm was scanned first to choose wavelengths for the maximum absorbance and the background. A serial dilution of BSA at 0, 2, 4, 6μg/mL was used to construct a standard curve (concentration versus absorbance). The crude protein concentration in each cell sample was calculated by converting the absorbance to concentration using the standard curve and multiplying by the proper dilution factor.

3.2.9 Experimental Design and Statistical Analysis

The experimental design for most of the cytotoxicity or antioxidant activity experiments was a strip block design. Each test solution was randomly assigned to the columns of the plate while each concentration of the test solution or each oxidation time was randomly assigned to the rows of the plates. The replications were done as columns or rows whichever was available. The test solution incubated with HBSS and tBHP were done on different plates but in the same positions. Whole experiments were replicated on different days with different random

assignments of test solutions and concentration levels or oxidation times. To analyze, proc mix in SAS version 8.2 (SAS Institute Inc., Cary, NC) was used (see Appendix I for SAS codes).

3.3 Results and Discussion

3.3.1 Emulsion Preparation

Figure 3.1 shows the HPLC chromatograms of γ -oryzanol, ferulic acid and α -tocopherol. The retention time of ferulic acid was 1.5 minutes and γ -oryzanol was 5-9 minutes and the three major components, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate were collected at 5.2-5.8, 5.8-6.3 and 6.3-6.8 minutes, respectively.

High concentration of γ -oryzanol and α -tocopherol stock solution (sterile) in ethanol could be stored in a freezer but the concentration increased when evaporation of ethanol occurred. Concentrations were tested by HPLC and additional filtered ethanol was added to the stock to obtain a concentration of 50mM and the concentrations were assured by HPLC. Otherwise, without the addition of ethanol, direct dilution from greater than 50mM stock would result in γ -oryzanol and α -tocopherol precipitation. This may imply the importance of ethanol acting as both solvent and emulsifier. Therefore, 2mM stock solution for cell culture experiments were obtained by diluting 50mM in HBSS-emulsion containing 500 μ M lecithin and 10mg/mL fatty acid free BSA, resulting in 4% ethanol. The stock solution higher than 2mM was difficult to prepare because γ -oryzanol higher than 50mM was difficult to dissolve in ethanol and higher concentration of emulsifiers, lecithin, BSA or other emulsifiers would be required. The γ -oryzanol and α -tocopherol emulsions were light milky color but without any suspended particles and could be stored in a refrigerator for at least two months without precipitation. Ultrasonication in water bath to homogenize before use was ideal.

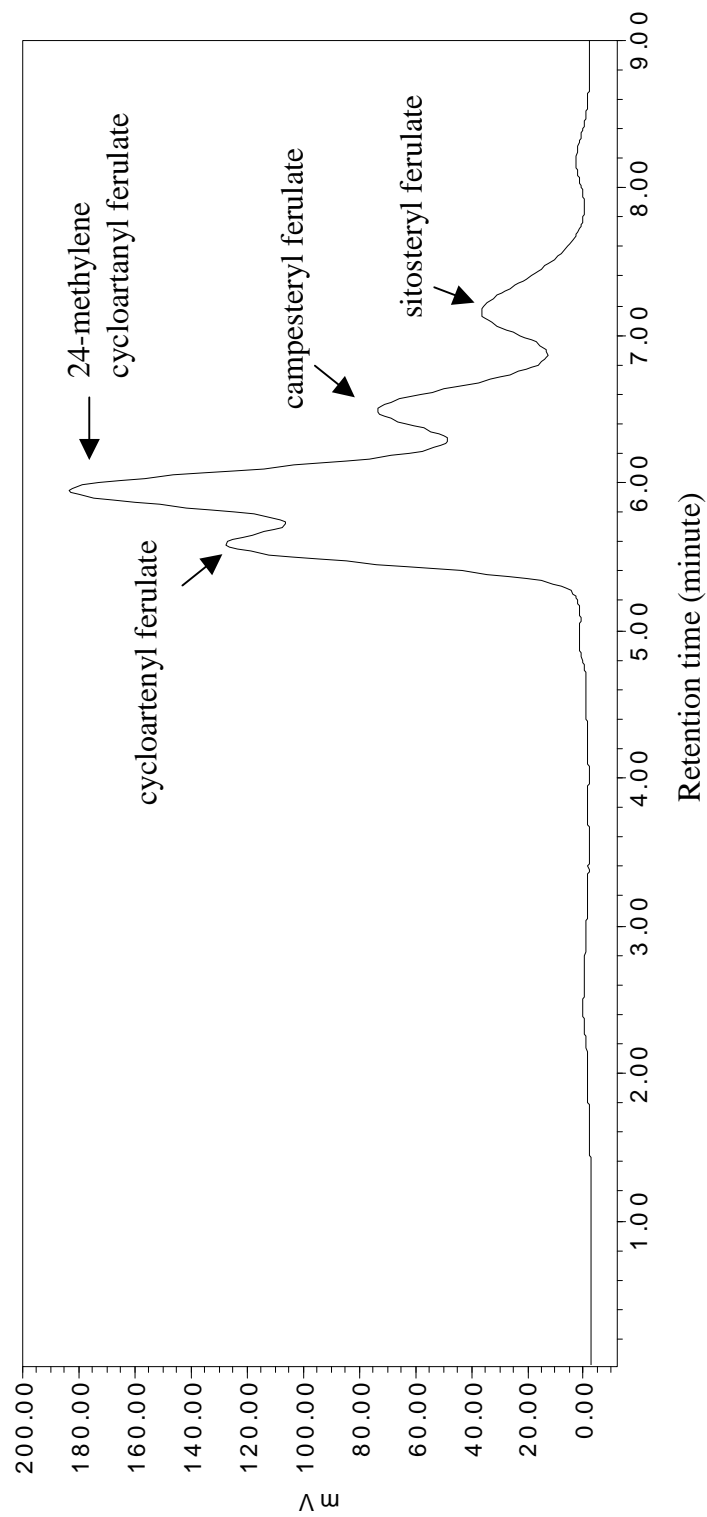


Figure 3.1(a). HPLC chromatogram of 0.1mM γ -oryzanol in mobile phase: methanol/acetonitrile/dichloromethane/acetic acid (50:44:3:3) at 1.4mL/min, absorbance at 330nm

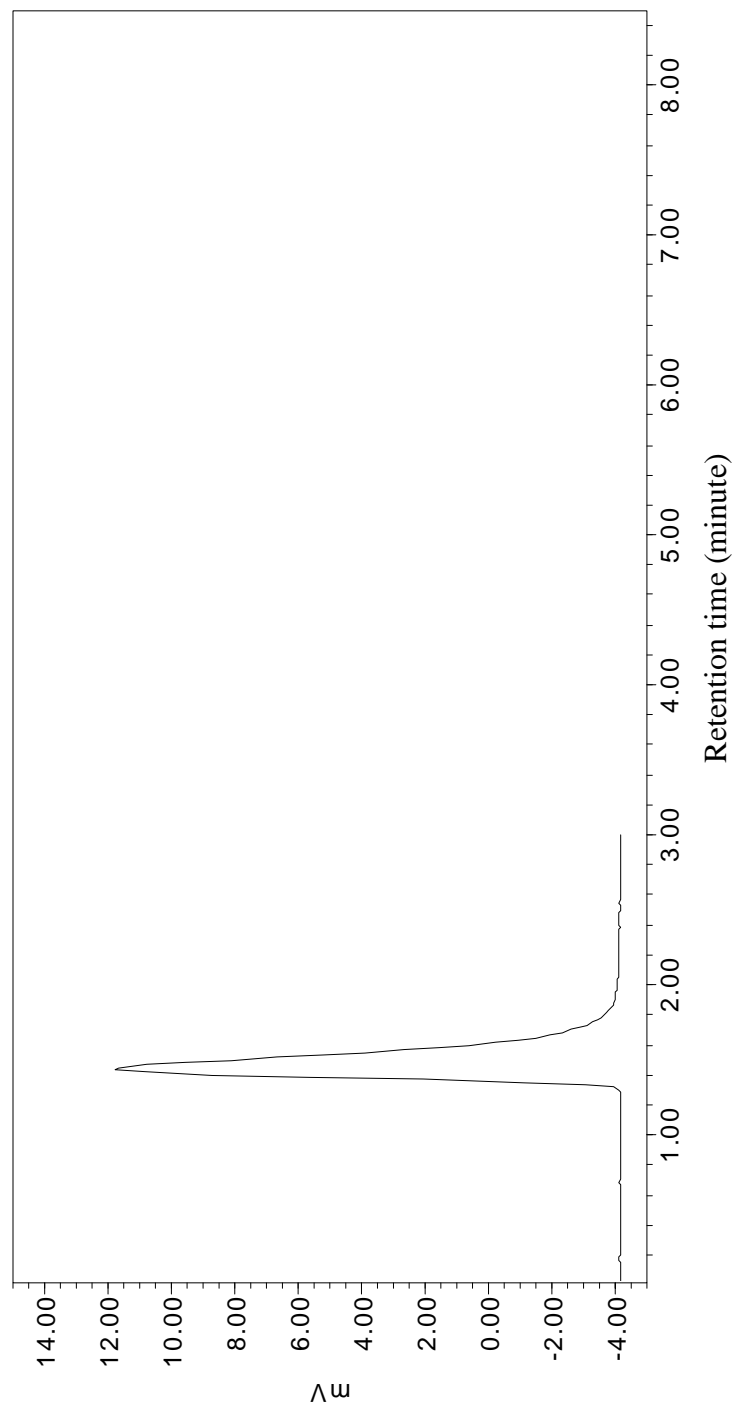


Figure 3.1(b). HPLC chromatogram of 0.1mM ferulic acid in γ -oryzanol mobile phase: methanol/acetonitrile/dichloromethane/acetic acid (50:44:3:3) at 1.4mL/min, absorbance at 330nm

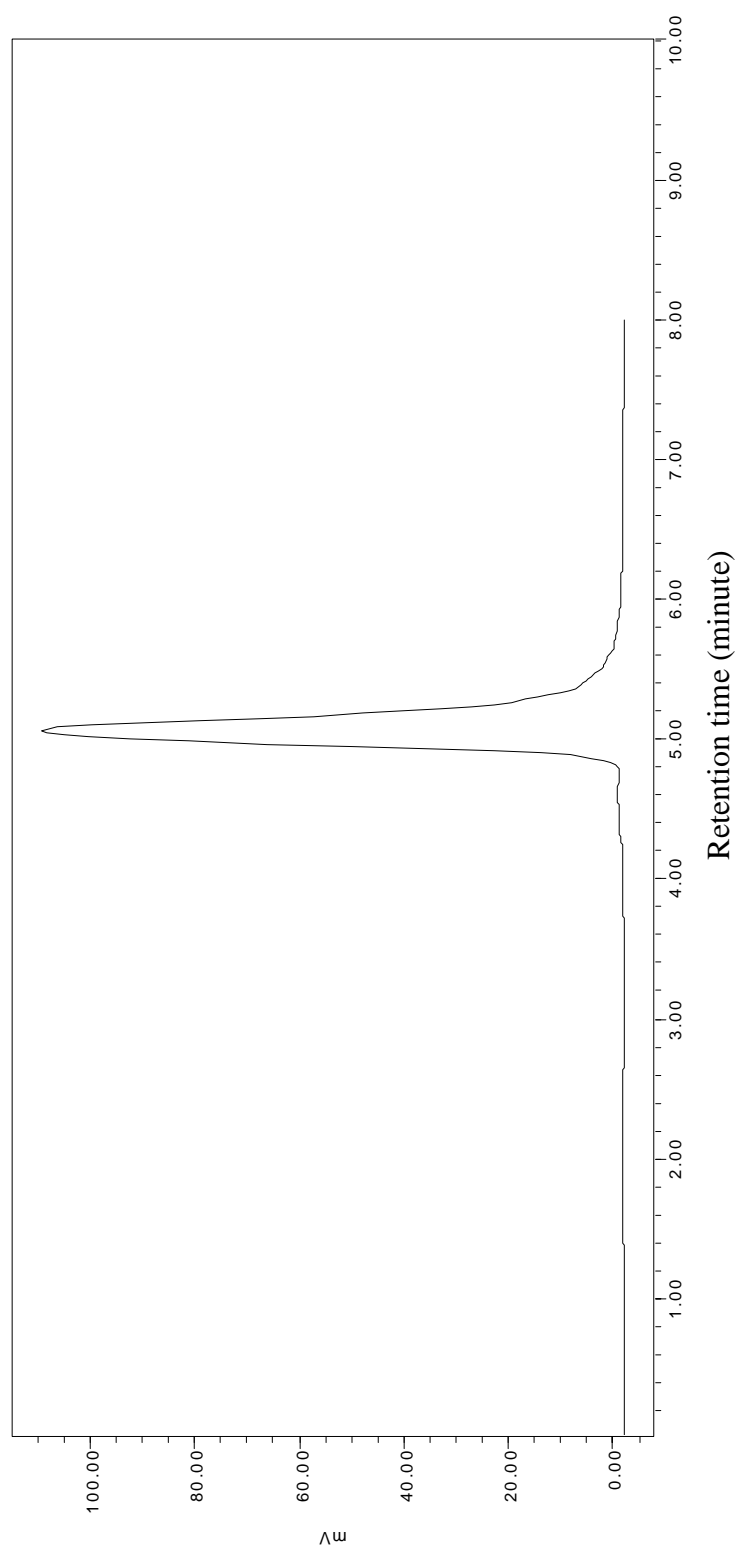


Figure 3.1(c). HPLC chromatogram of 0.1mM α -tocopherol in γ -oryzanol mobile phase: methanol/acetonitrile/dichloromethane/acetic acid (50:44:3:3) at 1.4mL/min, fluorescence detector, Ex: 290nm, Em: 330nm

3.3.2 Cell Selection, Cell Viability Assay and Cell Growth Curve

Besides SVEC4-10 cells, HUV-EC-C, a normal human umbilical vein endothelial cell line, and CPA, a normal bovine pulmonary artery endothelial cell line, were also cultured. However, both cell lines failed to grow well and required about 2 weeks to reach confluency. Thus, the SVEC4-10 cell line, which was available at the time, was used as a model to determine antioxidant activity. The SVEC4-10 cell line is an endothelial cell line from axillary lymph node vessels. The cells grow efficiently on plastic to a monolayer without the requirement of any other exogenous growth factors or matrix components. As for the method to measure cell viability, another assay to detect LDH released from injured cells was evaluated; however, the MTT assay was chosen because LDH reagent reacted with α -tocopherol and gave false positive and using it on SVEC4-10 cells showed low response signals (absorbance less than 0.1) whereas when using MTT, the response absorbance could reach as high as 0.6.

To construct a growth curve, SVEC4-10 cells were seeded starting at 4×10^5 cells/mL and counted everyday for 6 days. Unlike a conventional growth curve consisting of a lag phase, which is the time needed to recover from subculture and cell number remains the same, the cell populations decreased during the next day after subculture (Figure 3.2). This situation commonly occurs in some cell lines when cells recover from the stress at the beginning. Then the cell populations increased exponentially (day1-4), the log phase, until reaching the plateau phase, in which the cells reached confluency to form a monolayer and the cell growth slows down due to the limited nutrition and generated wastes (day4-5). However, some tumor cells, as in this case, can later accumulated and continue to grow for a few more days. To maintain a cell line, it would be preferable for the next passage at the end of the log phase to yield healthy and consistent cell cultures with good reproducibility. Therefore, to maintain consistency for further experiments,

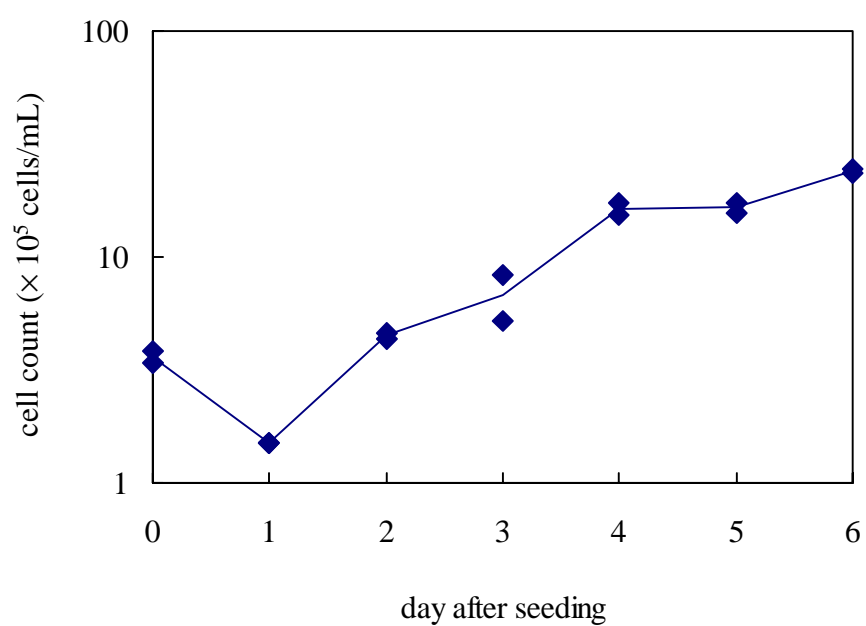


Figure 3.2. Growth curve of SVEC4-10 cells starting at 4×10^5 cells/mL after subculture (data shown were duplicates)

the SVEC4-10 cells were always plated at 4×10^5 cells/mL and used for antioxidant activity studies at the 3rd to 4th day, when they had reached confluency.

3.3.3 Cytotoxicity Studies of γ -Oryzanol, Ferulic Acid and α -Tocopherol Emulsion

3.3.3.1 Cytotoxicity Studies of Emulsifiers and Ethanol

Results in Figure 3.3 show that, statistically, there was a lecithin concentration main effect and a BSA concentration main effect but no interaction between lecithin and BSA. However, each concentration of BSA (1, 3, 5 or 7 mg/mL), regardless of the lecithin effect, and each concentration of lecithin (0.05, 0.1, 0.15 or 0.2mM), regardless of the BSA effect, were not significantly different from the control (HBSS) when incubated with cells for 4.5 hours. There was also no significant difference between each of the 36 lecithin and BSA (6 \times 6) combinations and the control, including the mixture of 0.25mM lecithin and 5mg/mL BSA in HBSS, which was used to prepare the emulsion of γ -oryzanol, α -tocopherol or ferulic acid in the later studies.

Since ethanol was one of the few solvents that could be used to dissolve γ -oryzanol, α -tocopherol or ferulic acid and was used for cell culture experiments, the effect of 2% ethanol in HBSS emulsion with 0.25mM lecithin and 5mg/mL BSA was studied. The results showed that, the cellular mitochondrial activity (93.03%) was not significantly different from the control (HBSS) (100%), when 2% ethanol HBSS-emulsion was incubated with SVEC4-10 cells for 4 hours (Figure 3.4(a)), while it significantly decreased to 77.02% when incubated for 22 hours ($p < 0.05$) (Figure 3.4(b)). Even though SVEC4-10 cells were more susceptible to 2% ethanol, it was necessary to use ethanol due to the difficulty in dissolving γ -oryzanol and α -tocopherol in aqueous culture solution. Therefore, in the further experiments, 2% ethanol in HBSS emulsion was always performed and referred to as the control for comparison.

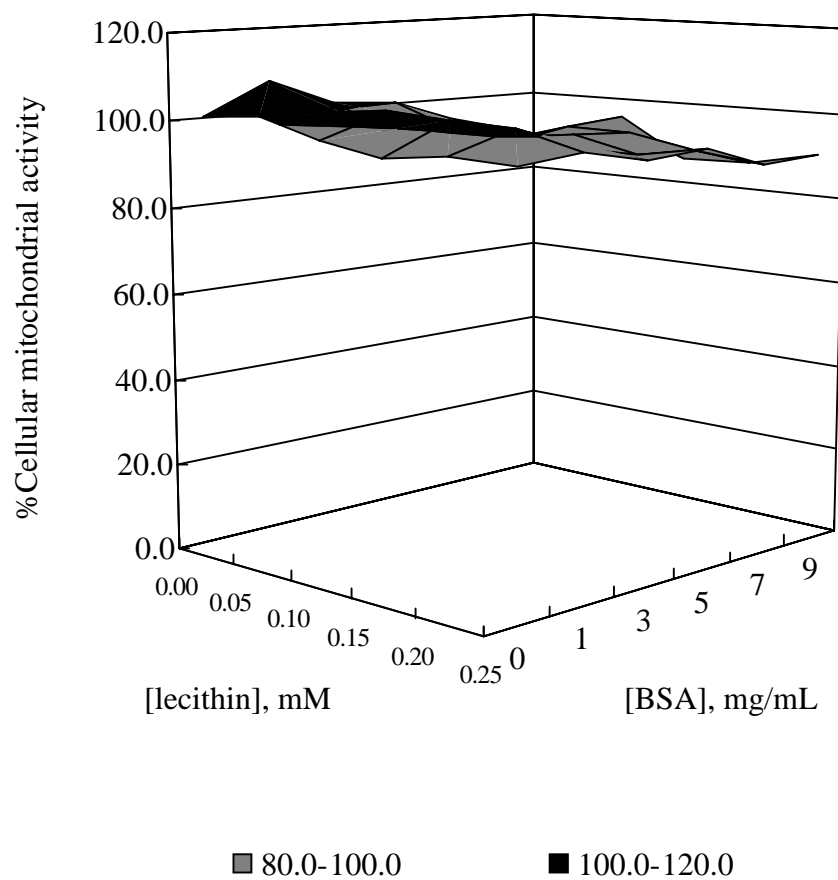
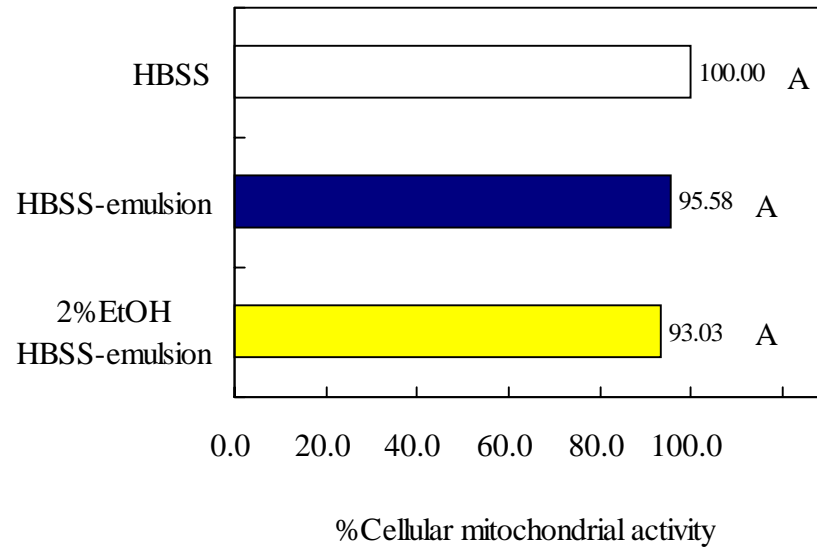


Figure 3.3. Cytotoxicity study of emulsifiers, lecithin and fatty acid free BSA incubated with SVEC4-10 cells for 4.5 hours at 37°C

(a)



(b)

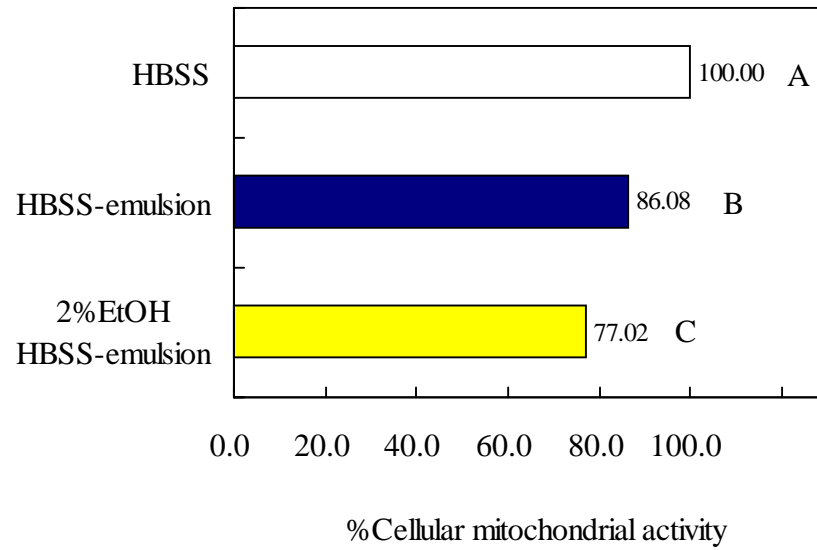


Figure 3.4. Cytotoxicity studies of 2% ethanol in HBSS-emulsion incubated with SVEC4-10 cells for (a) 4 and (b) 22 hours. Significant differences ($p<0.05$) are expressed by different letters.

3.3.3.2 Cytotoxicity Studies of γ -Oryzanol, Ferulic Acid and α -Tocopherol

First of all, the results did not show any false positives between γ -oryzanol, ferulic acid or α -tocopherol and MTT reagent, which means γ -oryzanol, ferulic acid or α -tocopherol if present would not interfere with the MTT assay. The cytotoxicity results showed that when incubated with SVEC4-10 cells for four hours, the effects of γ -oryzanol, ferulic acid and α -tocopherol on cellular mitochondrial activity were not all the same when averaged out for concentration effect (Figure 3.5(a)). γ -Oryzanol and α -tocopherol concentrations up to 1.0mM did not decrease cellular mitochondrial activity significantly while ferulic acid at 0.8 and 1.0mM did ($p<0.05$). Therefore, the highest concentrations of γ -oryzanol and α -tocopherol studied for their antioxidant activity would not exceed 1.0mM and 0.6mM for ferulic acid.

The results in Figure 3.5(b) showed that when incubated with cells for 22 hours, γ -oryzanol, ferulic acid and α -tocopherol up to 1.0mM did not decrease cell viability significantly. There was no concentration effect when averaged out for the effect of the type of test compound. However, to be conservative due to the fact that the overnight preincubation may be a stress to cells, 0.5mM was the highest concentration of γ -oryzanol, ferulic acid or α -tocopherol tested for their antioxidant activity.

Additionally, since the three major components of γ -oryzanol make up about 70-80% of total γ -oryzanol, the cytotoxicity of the three pure compounds was also tested. Each of the components up to 1.0mM was incubated with cells for 1 hour and up to 0.5mM for 22 hours. The results showed that incubation time of 1 hour did not significantly decrease %cellular mitochondrial activity (Figure 3.6(a)) and up to 1.0mM of each compound incubated with cells for 1 hour would later be studied for antioxidant activity. The results also showed that the incubation time of 22 hours did not cause cell death, except for 0.5mM campesteryl ferulate

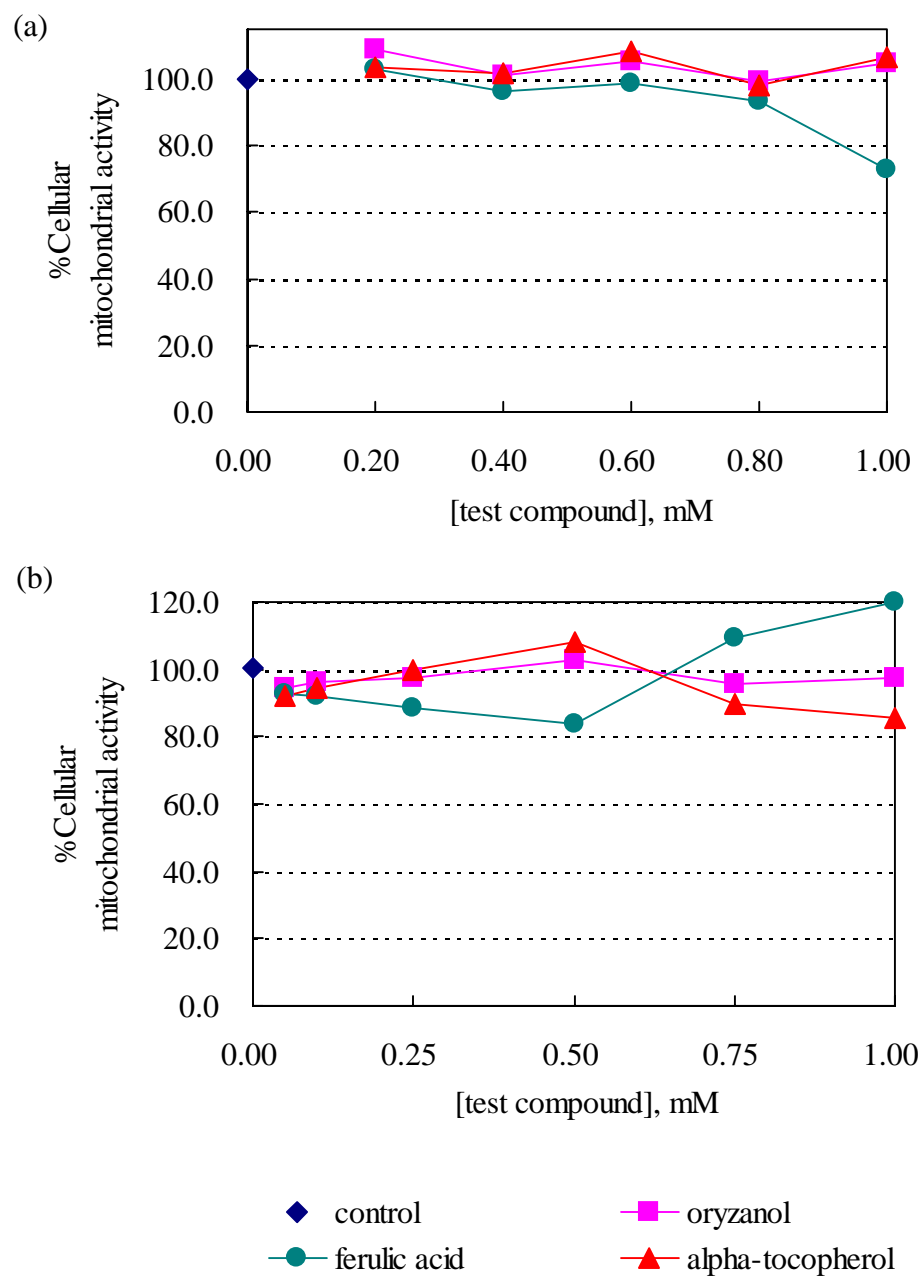


Figure 3.5. Cytotoxicity study of γ -oryzanol, ferulic acid and α -tocopherol incubated with SVEC4-10 cells for (a) 4 and (b) 22 hours

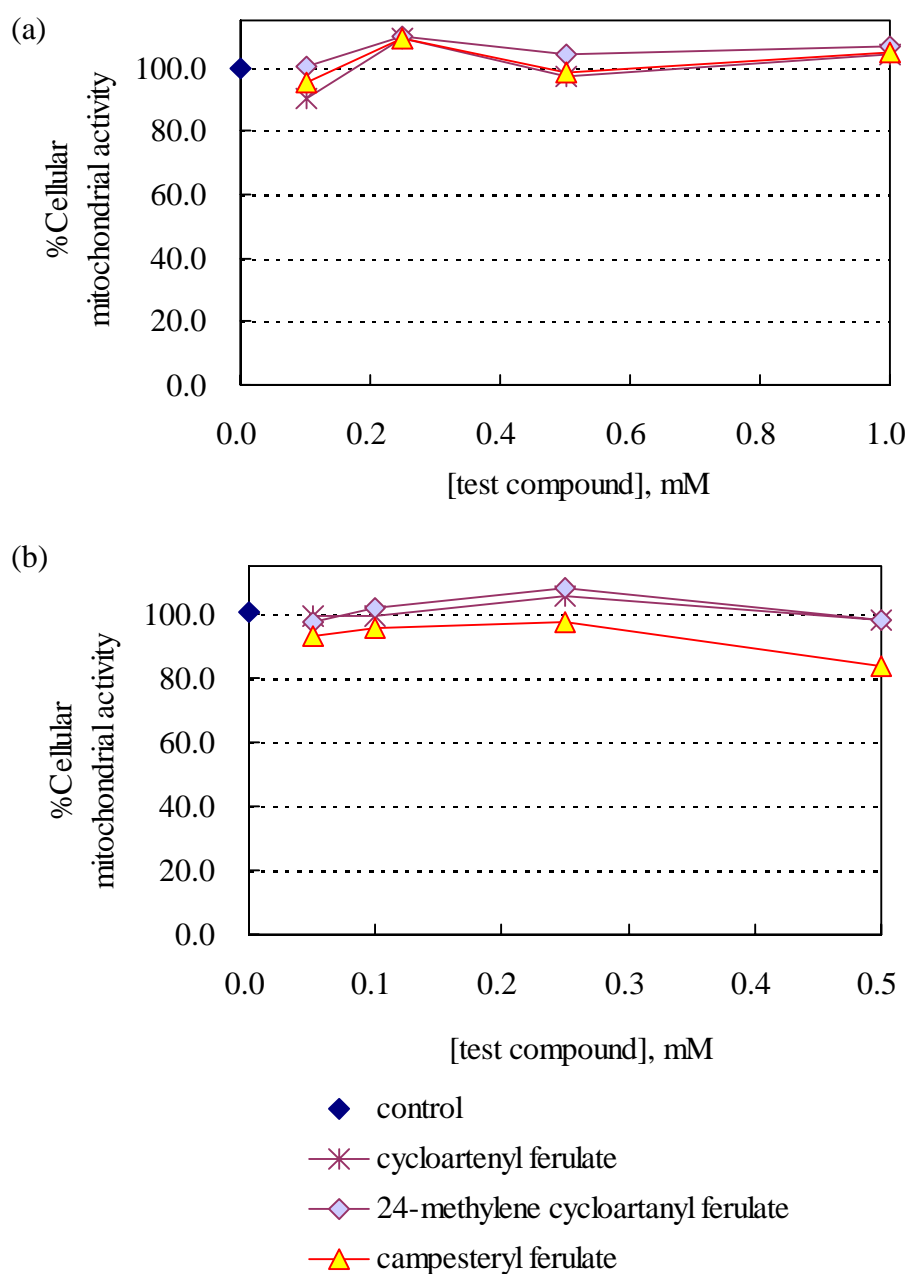


Figure 3.6. Cytotoxicity studies of the three major components of γ -oryzanol incubated with SVEC4-10 cells for (a) 4 and (b) 22 hours

(Figure 3.6(b)). Therefore, 0.5mM of each component would be the highest concentration studied for their antioxidant activity, except that 0.25mM was used for campesterol ferulate, if incubated with cells for 22 hours.

3.3.3.3 Cytotoxicity Studies of Interactions among γ -Oryzanol, Ferulic Acid and α -Tocopherol

The cytotoxicity of 0.50mM γ -oryzanol+0.50mM α -tocopherol, 0.50mM γ -oryzanol+0.30mM ferulic acid, 0.50mM α -tocopherol+0.30mM ferulic acid or 0.25mM γ -oryzanol+0.25mM α -tocopherol+0.20mM ferulic acid incubated with cells for 1 hour was studied and the result showed that no combinations diminished cell viability differently from the control (2% ethanol HBSS-emulsion) (Figure 3.7(a)). There was no effect of concentration levels regardless of type of test solution. Thus, the synergistic antioxidant activity among these test compounds at these concentration levels under this condition would be evaluated.

When incubated with cells for 22 hours, however, the results showed that γ -oryzanol+ferulic acid and α -tocopherol+ferulic acid at all concentrations significantly decreased cell growth compared to the control (Figure 3.7(b)). Therefore, only the synergistic antioxidant activity of γ -oryzanol+ α -tocopherol would be further studied.

3.3.4 The Effects of tBHP Concentration and Oxidation Time on SVEC4-10 Cellular Mitochondrial Activity

When cells were not preincubated with 2% ethanol HBSS-emulsion, statistically there were effects due to tBHP concentration, oxidation time and also concentration and time dependent interactions (Figure 3.8(a)). Regardless of the incubation time, 20mM of tBHP caused greater reduction in cellular mitochondrial activity than that caused by other levels of tBHP. Regardless of the concentration effect, oxidation for 60 minutes resulted in the greatest reduction among other shorter oxidation times. Furthermore, tBHP concentration of 12-20mM for 30-60

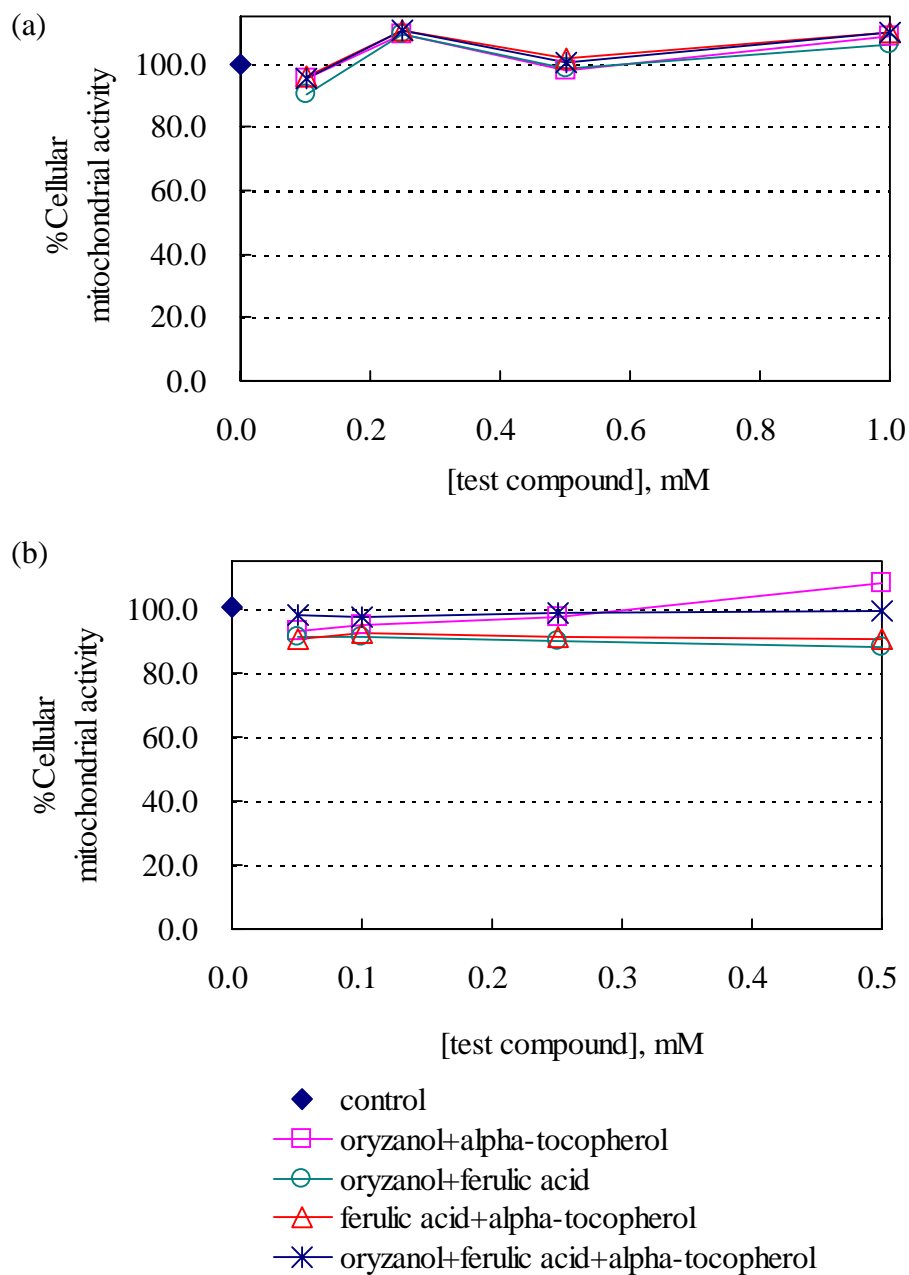


Figure 3.7. Cytotoxicity studies of interaction among γ -oryzanol, ferulic acid and α -tocopherol incubated with SVEC4-10 cells for (a) 4 and (b) 22 hours

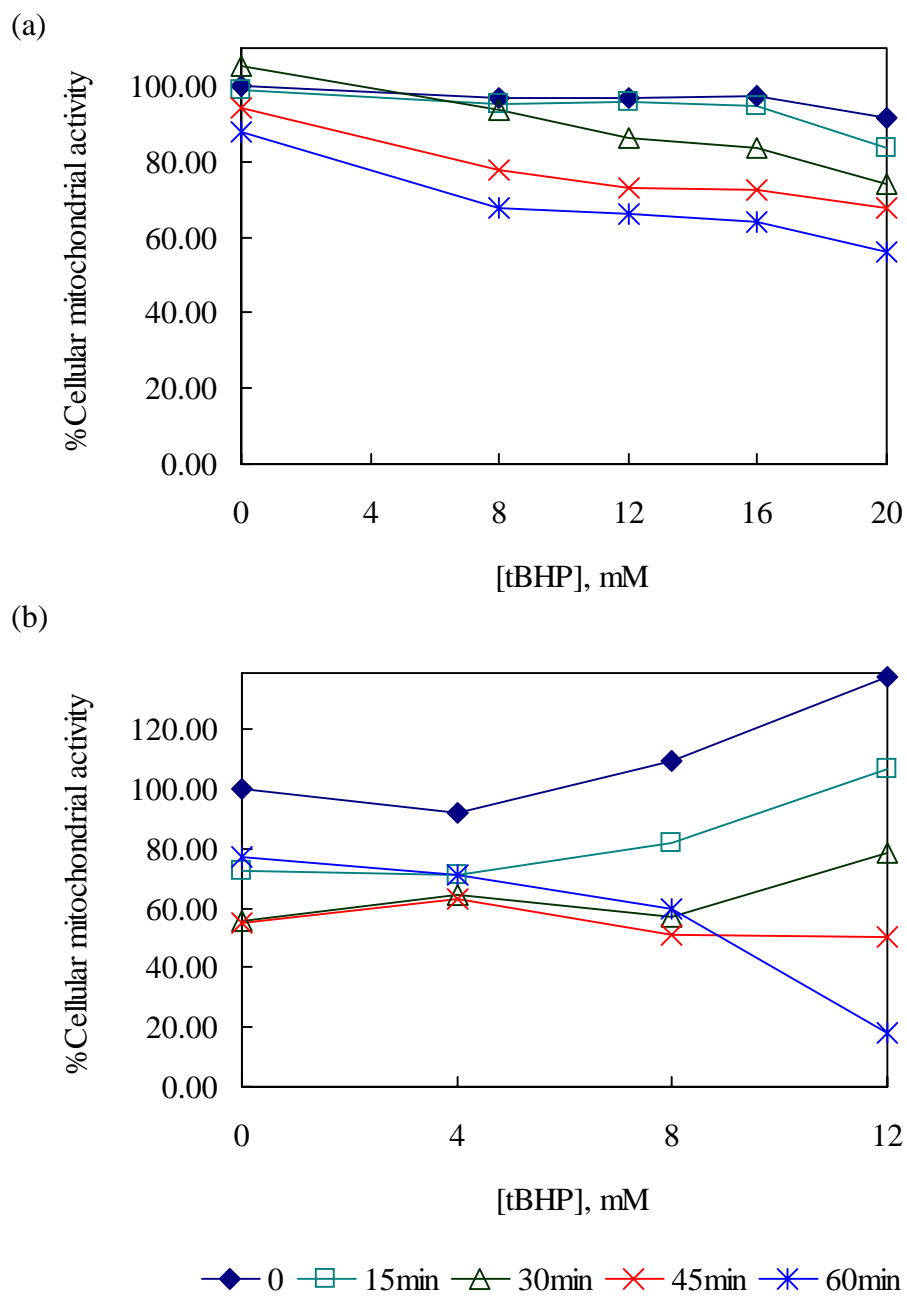


Figure 3.8. tBHP oxidation (0-60min) on SVEC4-10 cells (a) and (b) after preincubated with 2% ethanol HBSS-emulsion for 22 hours

minutes could significantly reduce cell viability compared to the control ($p<0.05$), but only 20mM injured cells significantly compared to any concentration level at any incubation time level. Thus, for further antioxidant activity experiments, tBHP oxidation conditions for SVEC4-10 cells without preincubation would be 20mM for 60 minutes.

For SVEC4-10 cells preincubated with test emulsions overnight, the oxidation conditions varied. Less oxidation for preincubated cells was needed to induce the same amount of cell injury. The results (Figure 3.8(b)) indicated that statistically there was no tBHP concentration effect, which indicates that with the oxidation time effect averaged out, there was no significant differences among any concentration level. However, overall there was an oxidation time effect (15-60 minutes in tBHP, regardless of concentration, significantly impaired cell viability compared to the control (HBSS) ($p<0.05$)). There was also an interaction effect between concentration and time ($p<0.05$). A tBHP concentration of 12mM at oxidation time of 60 minutes decreased cellular mitochondrial activity to a greater extent compared to any concentration level ($p<0.05$) but not at any time shorter than 45 minutes. Therefore, 12mM tBHP for 60 minutes was used later to study antioxidant activities of γ -oryzanol, ferulic acid and the three major components of γ -oryzanol compared to α -tocopherol which were preincubated with cells overnight.

There was an interesting characteristic of SVEC4-10 cell growth: when comparing the two formats (preincubation or not), cells seemed to revive more completely in response to oxidative stress after preincubation. Without oxidative stress, the cells grew significantly over time, which may mean that SVEC4-10 cells had a rapid metabolism, especially after preincubation with HBSS-emulsion. It has been reported that tBHP at very low concentration (10^{-5} mM) could actually stimulate cell growth on polyoma virus transformed baby hamster

fibroblasts and corresponding non-transformed cells (Burdon and Gill, 1989). The reason may be relevant to the inflammatory response to tissue replenishment following tissue damage due to oxidative stress. However, the tBHP concentrations used here to induce oxidative damage were much higher. More research is needed to further understand the mechanism of tBHP cytotoxicity. These results also provided another reason to always do HBSS experiments, in addition to tBHP, in order to compare tBHP/HBSS ratio to that of the control at 0 time.

These oxidation results may also imply that the oxidation conditions are critical in antioxidant activity studies. If the oxidation is not strong or long enough, the differences in cell viability between the treated and control may be too subtle to be detected. Also, if the oxidation is too strong or too long, the excess tBHP may damage all cells. In either situation small but real differences between each antioxidant activity could not be determined.

3.3.5 Antioxidant Activity and Possible Synergism of γ -Oryzanol, Ferulic Acid and α -Tocopherol

3.3.5.1 Antioxidant Activity of γ -Oryzanol, Ferulic Acid and α -Tocopherol at Different tBHP Oxidation Time

Antioxidant activity of γ -oryzanol, ferulic acid and α -tocopherol was determined relative to the effectiveness in preventing tBHP oxidative damage for 0, 15, 30, 45 or 60 minutes on mitochondria in SVEC4-10 cells. When γ -oryzanol, ferulic acid or α -tocopherol was simultaneously present with tBHP for 1 hour, the results (Figure 3.9(a) and Appendix II(a)) showed that the overall effect of γ -oryzanol, ferulic acid or α -tocopherol on reducing tBHP damage in cells, regardless of the oxidation time, was not significantly different from the control (2% ethanol HBSS-emulsion). There was a significant oxidation time main effect ($p < 0.05$), which means that the cell viability at each tBHP incubation time was different from each other being the highest at 0 minute and lowest at 60 minutes. There was no overall interaction between

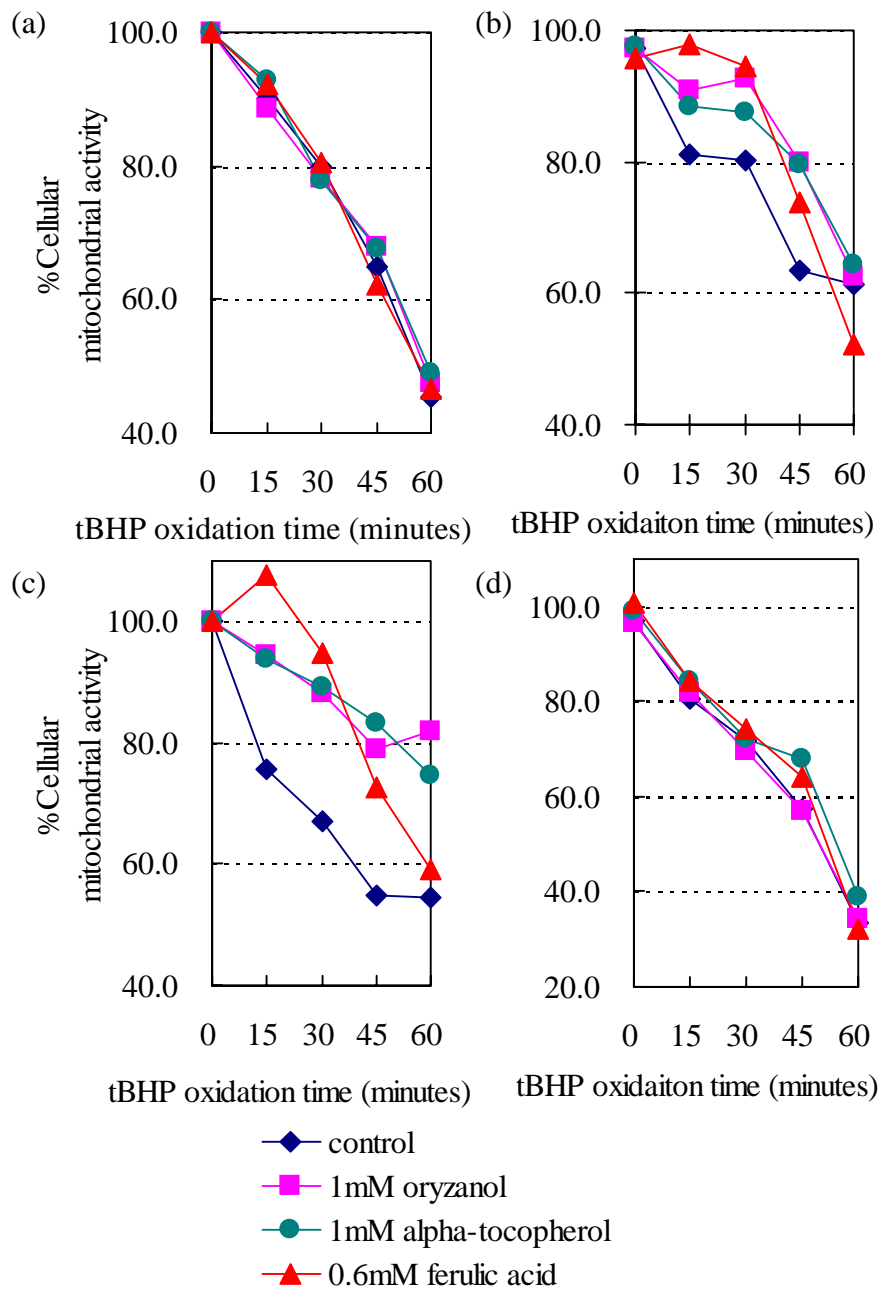


Figure 3.9. Antioxidant activity of γ -oryzanol, ferulic acid and α -tocopherol (a) with cells for 1 hour, (b) preincubated with cells for 22 hours then removed, (c) preincubated with cells for 22 hours then added tBHP for 1 hour, and (d) preincubated with tBHP for 22 hours then added to cells for 1 hour

the test solution and the oxidation time. All the test solutions were not significantly different from the control at oxidation time of 15, 30 or 45 minutes. Hence, it may imply that if present with tBHP simultaneously for 1 hour, γ -oryzanol, ferulic acid or α -tocopherol had no significant effect on preventing tBHP oxidative damage in cells.

When the cells were preincubated with γ -oryzanol, ferulic acid or α -tocopherol for 22 hours, washed and then oxidized by tBHP, the results showed that the type of test solution main effect was not significant, but the oxidation time main effect and the interaction of type \times oxidation time were significant ($p<0.05$) (Figure 3.9(b) and Appendix II(b)). When the oxidation time effect was averaged out, the cellular mitochondrial activity of any test solution was significantly greater than that of the control ($p<0.05$). When the test solution effect was averaged out, the effect of tBHP oxidation time at 60 minutes was significantly the greatest ($p<0.05$) but at 15 or 30 minutes was not significant. Statistically, if there is no interaction, the differences of antioxidant activity between any two test solutions should remain the same at any oxidation time. In this case, there was an interaction between the type of test solution and the oxidation time indicating that the test solution that had higher antioxidant activity at shorter time of oxidation was not always higher at longer time of oxidation and vice versa. At 15 and 30 minutes of oxidation, 0.50mM ferulic acid had the greatest antioxidant activity but had the lowest at 60 minutes. On the other hand, γ -oryzanol and α -tocopherol had an antioxidant activity not greater than ferulic acid at the early stage of incubation time but greater at the later stage. Therefore, it might imply that γ -oryzanol, ferulic acid and α -tocopherol all were associated with cells in some way and further protected cells from tBHP oxidative damage.

When γ -oryzanol, ferulic acid or α -tocopherol were preincubated with cells for 22 hours without washing, the results (Figure 3.9(c) and Appendix II(c)) showed that the type of test

solution main effect was not significant, but the oxidation time main effect and interaction of test type \times oxidation time on cell viability were significant ($p<0.05$). Oxidation time longer than 30 minutes was significantly different from 0 time ($p<0.05$), regardless of the type of the test solution. Similarly, the antioxidant activity of the control (2% ethanol HBSS-emulsion) was the lowest at any oxidation time. Ferulic acid had the greatest antioxidant activity, but was not significantly different from γ -oryzanol and α -tocopherol, at the oxidation time of 15 and 30 minutes while α -tocopherol and γ -oryzanol had a greater antioxidant activity at oxidation times of 45 and 60 minutes, respectively. It may be suggested that the antioxidant activity of γ -oryzanol, ferulic acid and α -tocopherol can extend to a greater degree if associated with the cells for 22 hours and continue to protect cells from tBHP oxidation. Also, ferulic acid may play a more important role in preventing tBHP damage at the early stage, then α -tocopherol and then γ -oryzanol later.

Finally, when test compounds were reacted with tBHP for 22 hours before being introduced to cells, the results (Figure 3.9(d) and Appendix II(d)) showed that there was a significant oxidation time main effect ($p<0.05$) and an interaction effect ($p<0.05$) but no test solution main effect. Regardless of the type of test solution, oxidation at any time was significantly higher than 0 time. Also, regardless of the oxidation time effect, γ -oryzanol, α -tocopherol and ferulic acid did not have an antioxidant activity significantly different from the control. These relationships held true at most oxidation times.

Thus, it may be suggested that the antioxidant activity mechanisms of γ -oryzanol, ferulic acid and α -tocopherol are different. When exposed to tBHP before cells, γ -oryzanol or ferulic acid had lower antioxidant activity than α -tocopherol. But when associated with cells, γ -oryzanol and ferulic acid can be more effective than α -tocopherol in preventing cell death. Thus, it may be

speculated that association with cells, probably on the cell membrane, is necessary for γ -oryzanol and ferulic acid to protect cells from tBHP oxidation. Comparing the chemical structure of γ -oryzanol with ferulic acid, the presence of the sterol or triterpene alcohol part in γ -oryzanol seems to alter the antioxidant activity of ferulic acid because γ -oryzanol did not always show higher antioxidant activity than ferulic acid.

3.3.5.2 Antioxidant Activity of The Three Major Components of γ -Oryzanol

As for the three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate, their antioxidant activity was also compared to α -tocopherol at different concentrations (0.05 to 0.5mM) by preincubation with the cells for 22 hours, followed by cell washing and 12mM tBHP oxidation for 1 hour. The results (Figure 3.10 and Appendix II(e)) showed that, neither the type of test solution main effect nor the concentration main effect was significant, but there was a significant interaction between the type of the test solution and the concentration level ($p < 0.05$). At each of concentration levels from 0.05 to 0.25mM, α -tocopherol tended to have an antioxidant activity higher than the control but not significantly ($p = 0.2786, 0.1901$ and 0.0784 , respectively), except at 0.5mM where 24-methylene cycloartanyl ferulate was the greatest and significantly greater than the control ($p < 0.05$). It may be suggested that α -tocopherol is an effective antioxidant even at concentrations as low as 0.05mM. On the other hand, γ -oryzanol did not have significant antioxidant activity at each concentration level tested but there was a trend towards increased activity in a concentration-dependent manner. Although not significant, cycloartenyl ferulate and campesterol ferulate at a concentration higher than 0.1mM tended to have a higher antioxidant activity than the control. At the lower concentration, the antioxidant activity of 24-methylene cycloartanyl

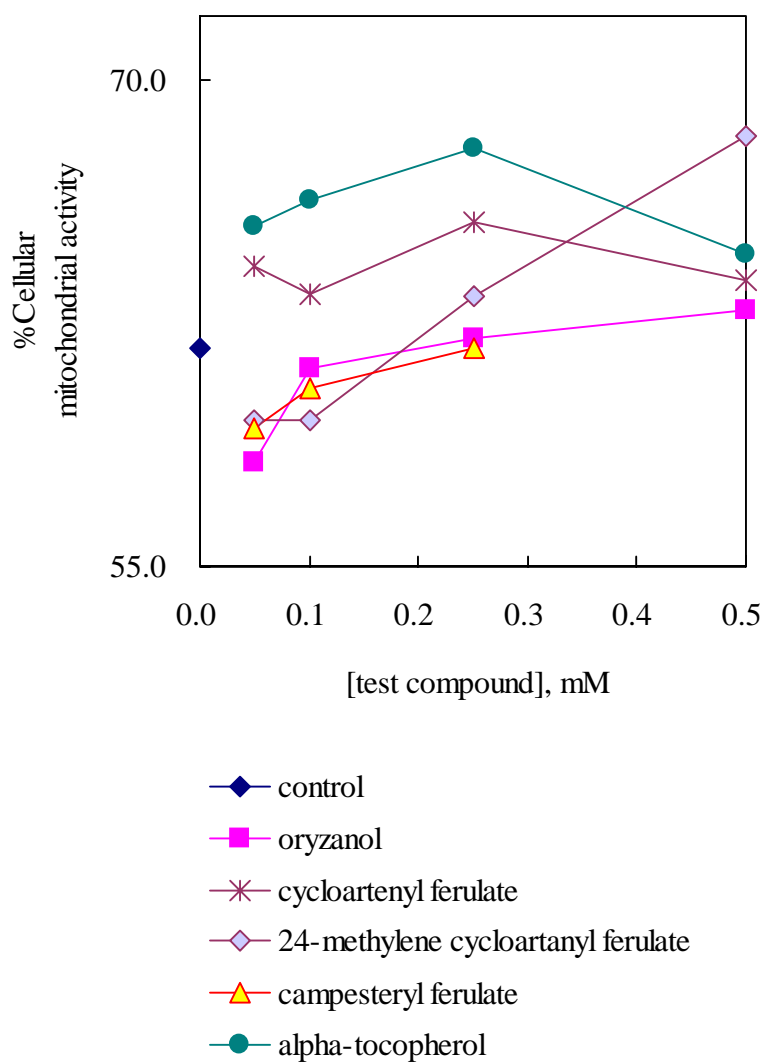


Figure 3.10. Antioxidant activity of the three major components of γ -oryzanol preincubated with cells for 22 hours and then tBHP oxidation for 1 hour

ferulate was not higher than cycloartenyl ferulate but was significantly higher at a concentration of 0.5mM ($p<0.05$).

The three major components all prevented tBHP oxidative damage in SVEC4-10 cells to a degree greater than γ -oryzanol. In another study, the three major components of γ -oryzanol were found to be more effective than γ -oryzanol in their inhibitory effect on 12-*o*-tetradecanoylphorbol-13-acetate-induced inflammation in mouse skin (Yasukawa *et al.*, 1998). More research on whether or not the anti-inflammatory effect is related to their antioxidant activity is needed. Since the three components constitute about 70-80% of total γ -oryzanol, it may be suggested that these three components may be more responsible for the antioxidant activity of γ -oryzanol than other minor components.

The antioxidant mechanism of ferulic acid has been evidenced from its potential of radical scavenging ability (Graf, 1992). Ferulic acid can form a phenoxy radical by donating a hydrogen atom to any reactive radical. The unpaired electron of ferulic acid radical can be delocalized across the entire molecule and thus becomes resonance stabilized. By comparing antioxidant activity and chemical structures, all of the γ -oryzanol components have the phenolic hydroxyl group in the ferulate portion, which can be a peroxy-radical scavenger. In addition, 24-methylene cycloartanyl ferulate has a methylene group at C-24 position attached to two alkyl groups while cycloartenyl ferulate has an alkene on C-24. The presence and the position of the double bond in the side chain of the terpene alcohol may also be related to its antioxidant activity.

24-Methylene cycloartanyl ferulate exhibiting a higher antioxidant activity than the other two components of γ -oryzanol and α -tocopherol was also observed in preventing cholesterol oxidation induced by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (Xu *et al.*, 2001).

This may suggest that among the components of γ -oryzanol, 24-methylene cycloartanyl ferulate had a higher antioxidant activity than the other components and, given a high enough concentration, it can even be more effective than α -tocopherol in preventing SVEC4-10 cell damage.

3.3.5.3 Synergistic Antioxidant Activity of γ -Oryzanol, Ferulic Acid and α -Tocopherol

When simultaneously present with tBHP and cells for 1 hour, the combination of γ -oryzanol+ α -tocopherol+ferulic acid (at concentration of 0.33mM, 0.33mM and 0.2mM, respectively) decreased the tBHP damage to cells, compared to the control, or 1.0mM γ -oryzanol or 0.6mM ferulic acid alone ($p<0.05$) or 1.0mM α -tocopherol ($p=0.1158$) (Figure 3.11(a) and Appendix II(a)). When preincubated with cells for 22 hours, even though not significant, the combination of 0.25mM γ -oryzanol+0.25mM α -tocopherol tended to have higher antioxidant activity than the control ($p=0.1747$), 0.50mM γ -oryzanol alone ($p=0.3463$) or 0.50mM α -tocopherol alone ($p=0.7170$) (Figure 3.11(b) and Appendix II(b)).

Based on these results, it may be suggested that a synergistic antioxidant activity of γ -oryzanol or ferulic acid with α -tocopherol possibly exists. α -Tocopherol has been studied extensively and is known for its function to inhibit lipid peroxidation by scavenging lipid peroxyl radicals to yield lipid hydroperoxides and a tocopheroxyl radical (Diplock *et al.*, 1998). The tocopheroxyl radical is less reactive and acts as a chain-breaking antioxidant. Tocopherol can be regenerated from the tocopheroxyl radical by ascorbic acid and glutathione. The synergistic effect of ascorbic acid on antioxidant activity of α -tocopherol was found to regenerate α -tocopherol because the ascorbyl radical not the tocopheroxyl radical was detected (Scarpa *et al.*, 1984). Therefore, it can be postulated that the synergistic antioxidant activity among γ -oryzanol, ferulic acid and α -tocopherol may be due to 1) the regeneration of α -tocopherol by γ -oryzanol

(a)

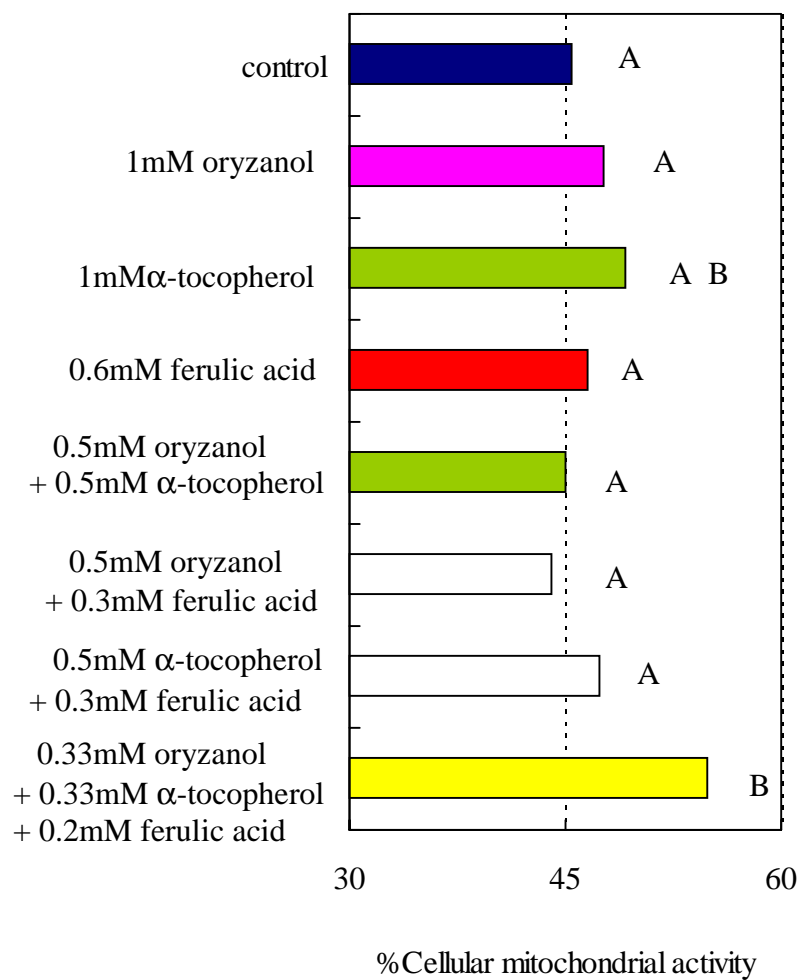


Figure 3.11(a). Synergistic antioxidant activity of γ -oryzanol, ferulic acid and α -tocopherol simultaneously incubated with cells and tBHP for 1 hour. Significant differences ($p < 0.05$) are expressed by different letters.

(b)

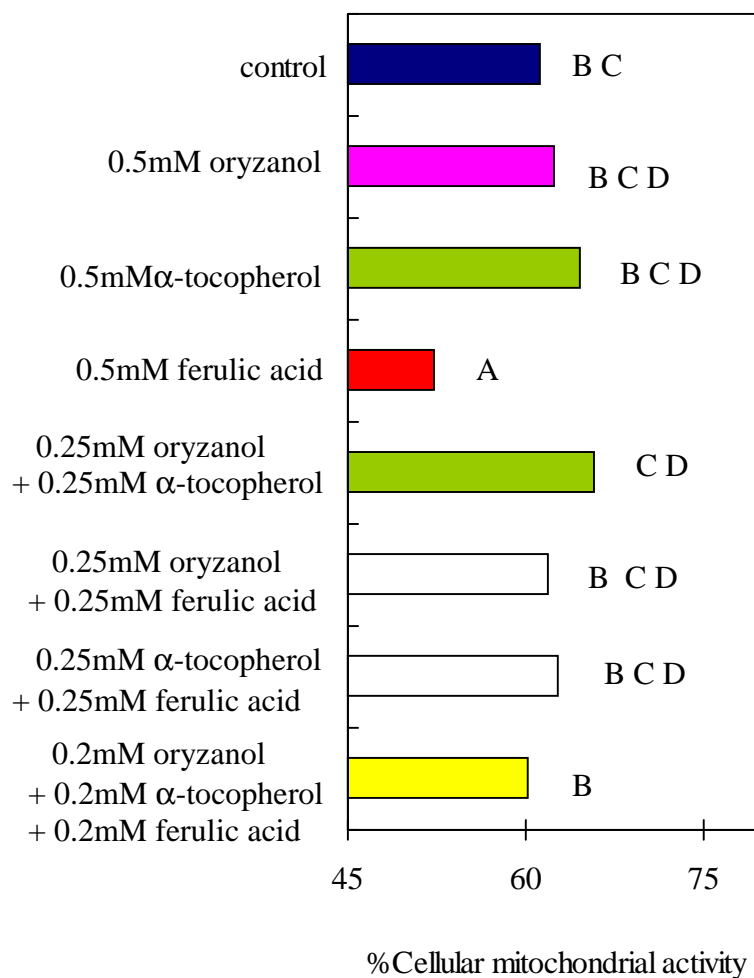


Figure 3.11(b). Synergistic antioxidant activity of γ -oryzanol, ferulic acid and α -tocopherol preincubated with cells for 22 hours then added tBHP oxidation for 1 hour. Significant differences ($p < 0.05$) are expressed by different letters.

and/or ferulic acid, 2) γ -oryzanol and/or ferulic acid spares α -tocopherol from radicals by quenching them, or 3) vice versa. Further investigation is required to understand the synergistic antioxidant mechanisms of γ -oryzanol, to identify the oxidized γ -oryzanol products and to develop the analytical method to detect them.

In a cell model using cells with fast metabolism such as SVEC4-10 cells, the cell viability is dynamic even in the control situation for a relatively short period of time, not to mention when incubated with any test substance and HBSS for a longer time, thus the cell viability can vary widely. These studies also support the approach to interpret cellular mitochondrial activity as a percentage of treated (tBHP) over control (HBSS) and compares it to that of 2% ethanol HBSS-emulsion at 0 treatment time. This mathematical adjustment may not be ideal but is fairly reasonable.

3.3.6 Lipid Peroxidation Assays

For the TBA-RS results, measuring the absorbances of the color products in standards and samples is recommended to scan absorbance spectrums for both standards and samples in every assay because the wavelengths for maximum absorbances can vary. Figure 3.12 showed that the maximum absorbance for TMP as standard was at 520nm but it was at 440-450nm for samples. The wavelength at 600nm was used to assess background interference in both standards and samples. The aim in this method would be to detect all substances reactive with TBA, which include not only malonaldehyde that produces a red pigment (at 520nm) but also alkanals, alkenals and 2,4-dienals that produce a yellow pigment (at 450nm). It has been suggested that it may be appropriate to measure both absorbances (Nawar, 1996). Thus, it may imply low specificity of this method.

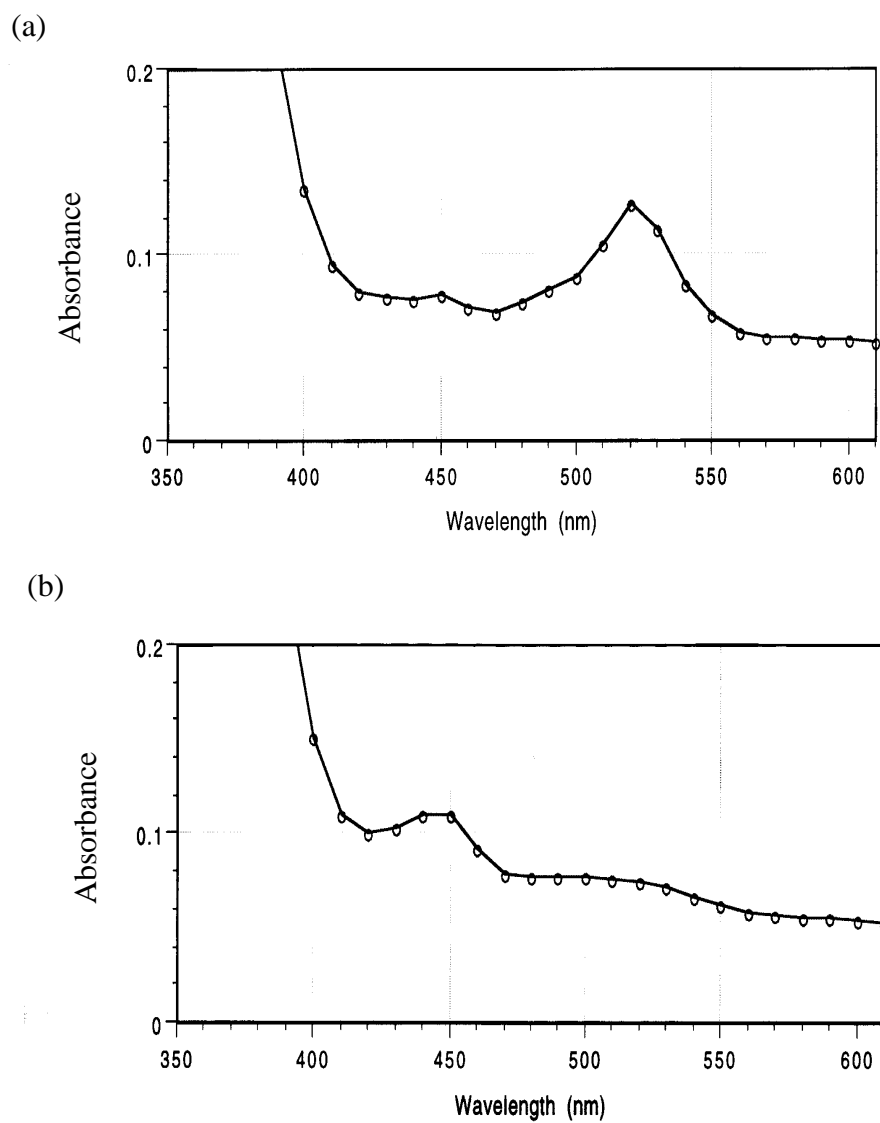


Figure 3.12. Spectrum scan from 350 to 610nm for (a) standard TMP (0.1µg/mL) and (b) TBA-reactive substances in cell samples treated with tBHP

The Bradford protein assay using Coomassie blue is the most widely used method and is commercially available as a kit. It measures protein content of cells to estimate total cellular material, which is often used as a denominator in expressions of, in this case, TBS-RS concentrations. It does not measure specific amino acids but is quite convenient. The maximum absorbances for BSA standards and samples were in a range from 595 to 640nm and absorbance at 790nm was used to assess background interference. Using SDS to solubilize protein or cells was not recommended because it could react with Bradford reagent.

When the SVEC4-10 cells were preincubated with γ -oryzanol, ferulic acid, α -tocopherol, their combinations or the three major components of γ -oryzanol for 22 hours and then washed and treated with HBSS or tBHP for 1 hour, the results in terms of excess TBA-RS produced during oxidation per unit protein showed that there was no significant difference among these test compounds and the control (2% ethanol in HBSS-emulsion) (Figure 3.13). Even though the same test solutions in the same conditions were studied for their effect on preventing cellular mitochondrial damage from tBHP oxidation, the results were found to lack agreement between them. Similar to this case, it also has been reported that some antioxidants can reduce TBA-RS formation but did not preserve cell viability (Rush *et al.*, 1985). The cytotoxicity of tBHP has been studied and postulated that lipid peroxidation may not be the main mechanism of tBHP cytotoxicity (Buc-Calderon *et al.*, 1991).

However, in this study the standard error for each test compound was relatively large, which implied poor reproductivity of this method. Furthermore, this method could not measure the TBA-RS released to solution during tBHP oxidation. Test solutions were always removed and cells were washed before testing because tBHP could react with TBA-RS reagent and give false positives.

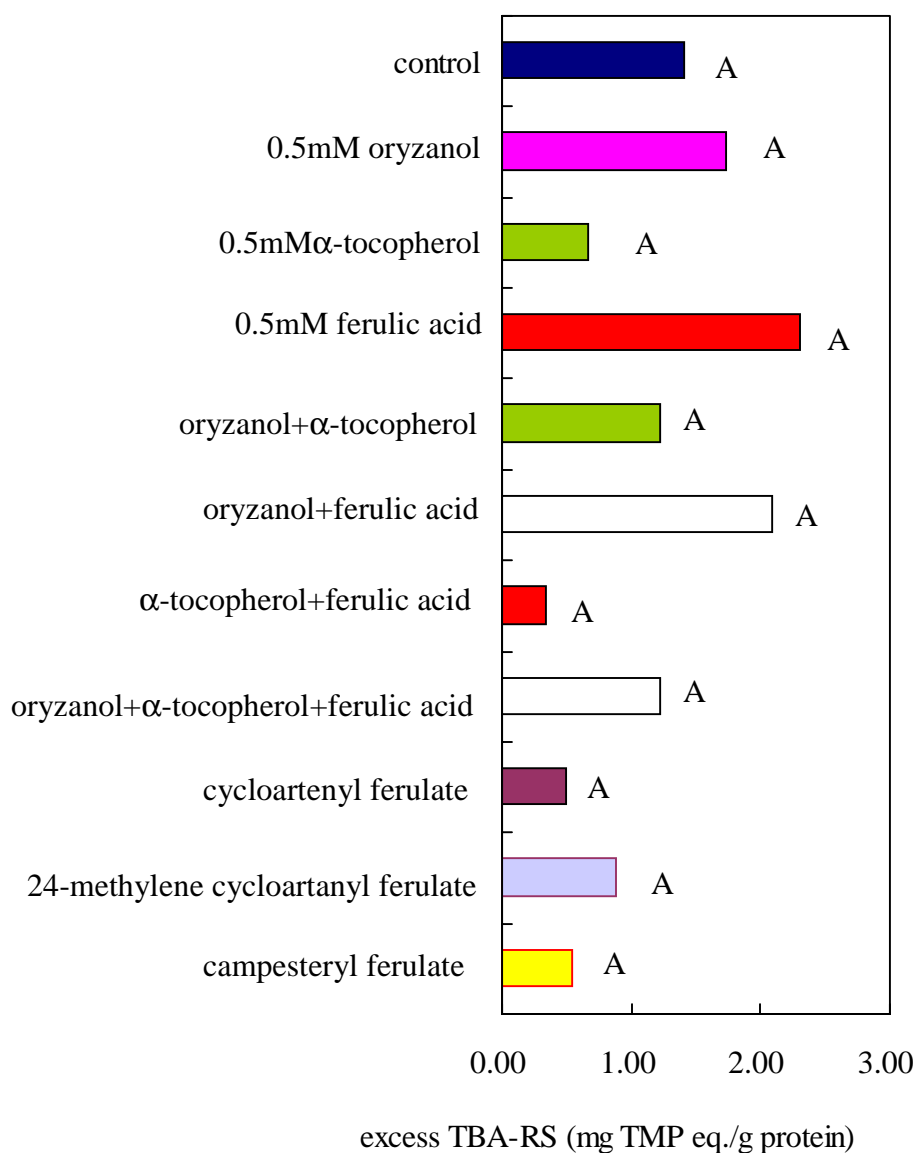


Figure 3.13. TBA-RS assay for antioxidant activity of γ -oryzanol, ferulic acid, α -tocopherol, their combinations and three γ -oryzanol major components preincubated with SVEC4-10 cells for 22 hours which were later treated with HBSS or 12mM tBHP for 1 hour. Significant differences ($p<0.05$) are expressed by different letters.

There were many disadvantages to the use of this TBA-RS method to study antioxidant activity of the compounds of interest, including poor reproducibility, low specificity and they are time and labor consuming. In addition, these TBA-RS experiments were scaled up using 8mL of each test solution and cells grown on 25cm² surface area flasks (equivalent to 100μL to surface area of a well in a 96-well plate), in order to amplify the response signals. Nevertheless, the results did not show any significance. Therefore, it is very difficult to draw conclusions without further investigation.

Another index of lipid peroxidation, conjugated diene formation, was also studied. However, the results showed that there was no peak detected at 234nm even in cells incubated with tBHP under conditions that would cause a marked decrease in cellular mitochondrial activity (Figure 3.14). Detecting lipid peroxidation has been known to be difficult due to the transient nature of lipid hydroperoxides and their decomposition (Aust, 1985). Therefore, it was not possible to distinguish the antioxidant activity of γ -oryzanol on lipid oxidation by introducing this method to the cell model developed for this study and whether or not the antioxidant activity of γ -oryzanol was responsible for preventing lipid peroxidation in cells was not delineated. A search in the future for other methods to approach this issue is necessary.

3.4 Conclusion

In this study, a cell model using SVEC4-10 mouse lymph endothelial cells, tBHP as oxidant agent and MTT for cell viability measurement was established and proven to be capable of distinguishing antioxidant activity of γ -oryzanol, ferulic acid, the three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate, from α -tocopherol. It was found that the SVEC4-10 cell line had a fast metabolism and consequently could be used to determine antioxidant activity of a test substance in a relatively

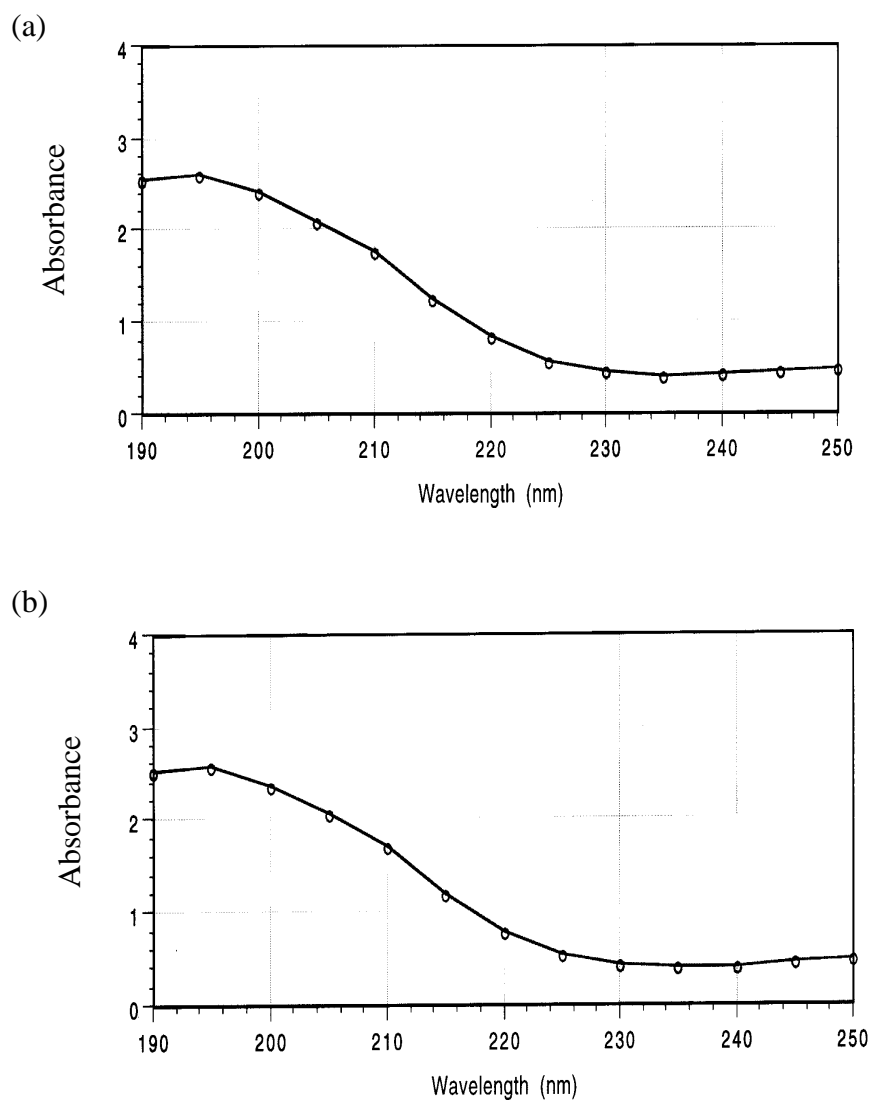


Figure 3.14. Spectrum scan from 190 to 250nm for conjugated diene formation from chloroform/methanol/H₂O extraction of SVEC4-10 cells treated with (a) HBSS or (b) tBHP oxidation for 1 hour

rapid manner. The critical and vital parameters relied on were 1) the emulsion preparation of hydrophobic compounds for cell model, 2) the consistent management of cell culture ready for antioxidant activity experiments, 3) the control of tBHP oxidation conditions such as concentration and time combinations and 4) the selection of cell viability detection methods compatible with the cell line and the test substances.

In summary, γ -oryzanol and ferulic acid did possess antioxidant activity, in terms of preventing cellular mitochondrial damage due to tBHP oxidation. When preincubated with the SVEC4-10 cells, ferulic acid had higher antioxidant activity than γ -oryzanol and α -tocopherol at the early stage of oxidation (0 to 30 minutes) while γ -oryzanol and α -tocopherol tended to have higher antioxidant activity than ferulic acid at the late stage of oxidation (30 to 60 minutes). The three major components of γ -oryzanol generally had higher antioxidant activity than γ -oryzanol and in some situations presented more effective antioxidant activity than α -tocopherol. Among these three major components of γ -oryzanol, 24-methylene cycloartanyl ferulate was found to be relatively more effective. A synergistic antioxidant activity among γ -oryzanol, ferulic acid and α -tocopherol was also found when simultaneously present with tBHP for one hour.

CHAPTER 4. HYPOCHOLESTEROLEMIC CAPACITY EXPERIMENTS USING AN *IN VITRO* HUMAN INTESTINAL CELL MODEL

4.1 Introduction

Another potential functionality of γ -oryzanol, hypocholesterolemic capacity, has been reported in a range of human and animal studies including rats and monkeys, which showed promising results for γ -oryzanol in reducing serum total, free, esterified and liver cholesterol levels. This research is the first attempt to develop an *in vitro* cell model using human intestinal cells and use it to demonstrate the possible mechanisms of hypocholesterolemic capacity of γ -oryzanol.

Since the digestive, absorptive and metabolic fate of γ -oryzanol is not clearly delineated and in view of the γ -oryzanol effect on enhancing cholesterol excretion, the potential mechanism by which γ -oryzanol decreases cholesterol uptake may be related to its action in the lumen of the gastrointestinal tract. In the human intestines, there are two sources of cholesterol. Typically one-third comes from diet (exogenous source) and the remaining two-thirds from bile (endogenous source). It has been demonstrated that decreasing cholesterol uptake can lead to decreasing serum cholesterol. Therefore, this study focused on γ -oryzanol effects in the intestines to decrease cholesterol uptake.

In order to approach this goal, one objective of this study was to develop an *in vitro* cell model using a human intestinal cell line to study cholesterol uptake and use a gas chromatography (GC) coupled with mass spectrometry (MS) to analyze cholesterol. In addition, since dietary cholesterol consists of two forms, free and esterified, the γ -oryzanol effect on the stability of micelles containing both forms of cholesterol or the availability of total cholesterol for uptake would be studied. Finally, the pancreatic enzymes, cholesterol esterase (CEase) and

phospholipase A₂, present in the intestines have been reported to promote cholesterol uptake (Mackay *et al.*, 1997) and CEase is also responsible for hydrolyzing cholesteryl esters. Therefore, the possibility that γ -oryzanol inhibits or otherwise interferes with the activity of these enzymes would also be investigated.

4.2 Materials and Methods

4.2.1 Materials and Reagents

γ -Oryzanol was obtained from crude rice bran oil using a preparative normal-phase high performance liquid chromatography (HPLC) and crude rice bran oil was extracted from milled rice bran according to the method developed by Xu and Godber (1999). The preparative HPLC system consisted of a Waters (Milford, MA) Prepak[®] RCM base connected with two cartridges (each 25mm×10cm Prep Nova-Pak HR silica with particle size 6 μ m) and a Guard-Pak insert, a U6K manual injector, a 510 pump and a Lambda-Max 481 LC Spectrophotometer (wavelength 330 nm). Mobile phase was methanol with a constant flow rate at 15 mL/min and 2mL of the crude oil were injected each time. The eluate containing γ -oryzanol was collected (25-30min) and the solvent was evaporated.

Cholesterol, cholesteryl oleate, oleic acid, 1-monoolein, lecithin, sodium taurocholate, MTT assay kit, Bradford reagent, Hank's balanced salts, porcine pancreatic cholesterol esterase, phospholipase A₂ and trimethylsilylation reagents (N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1)) were purchased from Sigma, St. Louis, MO. Tris(hydroxymethyl)aminomethane was purchased from Amresco, Solon, OH. HCl was purchased from Mallinckrodt, Inc., Paris, KY. NaOH, hexane and isopropanol were purchased from Fisher Scientific, Fair Lawn, NJ. Ethanol was USP absolute 200 proof purchased from AAPER Alcohol and Chemical Co., Shelbyville, KY. Culture plates were purchased from

Corning Inc., Corning, NY, and the sterile disposable syringe filter was 0.2 μ m PES purchased from Whatman, Clifton, NJ.

Tris-HCl buffer (0.1M) was prepared by dissolving 1.2114g of Tris(hydroxymethyl)aminomethane in about 90mL of distilled deionized water, adjusting pH to 7.5 with 10N of HCl and diluting to 100mL volume with water.

4.2.2 Micelle Preparation

A modified method from Nagaoka *et al.*, (1999), was used to prepare micelles containing cholesterol, cholesteryl oleate and/or γ -oryzanol in aqueous solutions. Cholesterol or cholesteryl oleate at a concentration of 0.2mM with or without 0.2mM γ -oryzanol was dissolved in hexane:isopropanol (3:1) with 1mM oleic acid, 0.5mM 1-monolein and 0.6mM lecithin (final concentration). The solvent was evaporated using a vacuum evaporator (45°C, CentriVap Console, Labconco Inc., Kansas City, MO) and then 6.6mM sodium taurocholate in buffer or culture medium was added. Each solution was ultrasonicated (Sonic Dismembrator, Fisher Scientific, Fair Lawn, NJ) at output power of 10 watts for 45 minutes in an ice bath in order to ensure a clear solution free of precipitation. For cell culture experiments, micellar solutions were filtered through a 0.2 μ m filter for sterilization before being applied to cells. The cholesterol and cholesteryl oleate concentrations in the filtrate were later extracted and determined by GC/MS to ensure that cholesterol or cholesteryl oleate did not precipitate and that the micelle formation was successful.

4.2.3 Effect of γ -Oryzanol on Micellar Solubility of Cholesterol and Cholesteryl Oleate

Cholesterol and cholesteryl oleate solubility in micelles with γ -oryzanol was measured by adding 10 μ L of ethanol or 10mM γ -oryzanol in ethanol to 990 μ L of 0.1mM of cholesterol or cholesteryl oleate micellar solution (final γ -oryzanol concentration was 0.1mM and ethanol was

1%). The mixture was vortexed, incubated in a shaking water bath at 37°C for 24 hours and then ultracentrifuged (Beckman TL100 Ultracentrifuge, Beckman Instruments, Inc., Palo Alto, CA) at 100,000×g at 37°C for 1 hour. The supernatant (500μL) was extracted to determine the γ -oryzanol concentration by HPLC. The remaining samples were dried under vacuum for cholesterol or cholesteryl oleate analysis by GC/MS.

4.2.4 Study of γ -Oryzanol Inhibition on CEase-Facilitated Cholesteryl Oleate Hydrolysis

To study γ -oryzanol effect on CEase-facilitated cholesteryl oleate hydrolysis, first of all, the relationship between the substrate (cholesteryl oleate) concentration and the hydrolytic enzyme (CEase) concentration had to be determined since it could affect the reaction rate, by which an understanding of the enzyme kinetics and the possible inhibition mechanism by γ -oryzanol could be obtained. Cholesteryl oleate micellar solution (1mL of 0.2mM in 0.1M Tris-HCl buffer, pH 7.5) was incubated with a serial of concentrations of CEase from 0, 0.003, 0.03 to 30μg protein/mL in a shaking water bath at 37°C for 5 minutes. After incubation, solvent extraction was immediately performed, followed by GC/MS to determine the concentration of cholesterol released from hydrolysis. The lowest CEase concentration that could result in a detectable amount of cholesterol significantly different from the control emulsion without CEase would be used to study the γ -oryzanol effect on CEase activity.

To study γ -oryzanol effect on CEase inhibition, 1mL micellar solution containing cholesteryl oleate at concentrations of 0.05, 0.2 or 2mM with or without 0.1mM γ -oryzanol was incubated with CEase at 37°C for 5 minutes, immediately followed by solvent extraction and GC/MS determination of the amount of cholesterol released. The results would be expressed in a Michaelis-Menten plot (initial reaction rate versus substrate concentration) and a double-reciprocal, or Lineweaver-Burk, plot (reciprocal of initial reaction rate versus reciprocal of

substrate concentration). To determine the type of inhibition, if present, and the SAS system (version 8.2, SAS institute, Cary, NC) was used to compare the regression curve of the with γ -oryzanol effect to the without γ -oryzanol curve.

4.2.5 Cholesterol Uptake by Human Intestinal Cells *In Vitro*

4.2.5.1 Cell Culture

Human large intestinal cell line C2BBel (ATCC CRL-2102) was used to study cholesterol uptake. The cell line was maintained in 25-cm² flasks with vent caps. To subculture, cells were washed three times using 0.25% trypsin (1mL each time). On the third time cells were kept in the trypsin solution for 10 minutes at 37°C. After cells detached, new medium (90% DMEM with 4mM L-glutamine, 1.5g/L sodium bicarbonate and 4.5g/L glucose and supplemented with 0.01mg/mL human transferrin; 10% fetal bovine serum) was added and the cell suspension was split into new 25-cm² flasks (split ratio 1:3). For cholesterol uptake experiments, the cell suspension was diluted in new medium to 2×10^5 cells/mL and 1mL was transferred to each well (area=2cm²) of the 24-well culture plates, which resulted in 10^5 cells/cm². For cytotoxicity studies, 96-well plates were used and 100 μ L of the cell suspension was cultured in each well. Cell count was performed at each subculture to ensure consistency for each experiment. Cells were used for experiments when they reached confluency (about the 4th day after subculture).

4.2.5.2 Cytotoxicity Studies of Micellar Solutions Using an MTT Assay

Before being applied to the cells for the study of cholesterol uptake, all micellar solutions were investigated for their cytotoxicity. After reaching confluency in 96-well culture plates, the cells were gently washed using Hank's balanced salt solution (HBSS) twice and 100 μ L/well of each test solution was applied for 2 hours at 37°C. After incubation, the test solutions were

discarded and the cells were gently washed again using HBSS, followed by a 3-(4,5-dimethylthiazol-yl)-2-5-diphenyltetrazolium bromide (MTT) assay to measure cell viability in terms of the activity of mitochondrial dehydrogenases in living cells. The cells were incubated with 40 μ L/well of 1.25mg/mL MTT in HBSS, which is yellow in color, at 37°C in the dark for 2 hours. The mitochondrial dehydrogenases of viable cells cleaved the tetrazolium ring and yielded purple formazan crystals, which are insoluble in aqueous solution. Then 100 μ L/well of MTT solubilization solution containing 10% Triton X-100 plus 0.1N HCl in anhydrous isopropanol was added to solubilize purple formazan crystals. Pipetting up and down at least 100 times helped solubilization in dense culture situation. Absorbances at 570nm for color development and at 690nm for background were read using a microplate spectrophotometer (SPECTRAmax® PLUS, Molecular Devices Co., Sunnyvale, CA). An increase or decrease of cell numbers after exposure to a test solution would correspond to a concomitant change in the amount of purple formazan formed or in the optical density at absorbance 570nm. The cell viability under exposure to a test compound was expressed in %cellular mitochondrial activity, which was the ratio of absorbance reading difference between 570nm and 690nm of test compound ($A_{570} - A_{690}$) to that of the control ($A_{570}^o - A_{690}^o$):

$$\% \text{Cellular mitochondrial activity} = \frac{A_{570} - A_{690}}{A_{570}^o - A_{690}^o} \times 100\%$$

4.2.5.3 Study of Cholesterol Esterase or Phospholipase A₂-Promoted Cholesterol Uptake by Human Intestinal Cells *In Vitro*

First of all, the cytotoxicity of 0.1mM cholesterol with or without CEase or phospholipase A₂ up to 100 μ g protein/mL was studied. The concentrations of each enzyme that did not affect the cell viability significantly would be used to investigate whether or not these two enzymes could facilitate cholesterol uptake into cells as stated by Mackay *et al.*, 1997, and if

so, whether or not γ -oryzanol could affect the cholesterol uptake facilitated by these two enzymes.

After reaching confluency, the cells were gently washed with HBSS twice and then incubated with 1mL of 0.1mM cholesterol micellar solution with or without each enzyme at various concentrations up to 100 μ g protein/mL for 2 hours at 37°C. After incubation, the test solutions were pipetted out and the cells were gently washed again with HBSS twice, followed by cell lysis for Bradford protein assay and cholesterol analysis as described in Section 4.2.7.2.

4.2.6 Effect of γ -Oryzanol on Cholesterol Uptake by Human Intestinal Cells *In Vitro*

4.2.6.1 Effect of γ -Oryzanol on Micellar Solubility of Cholesterol

Before the uptake experiments, cytotoxicity of cholesterol micellar solution with or without γ -oryzanol was studied. Cells were incubated with 100 μ L of 0.1mM cholesterol or a mixture containing 0.1mM cholesterol and 0.1mM γ -oryzanol for 2 hours at 37°C, followed by cell washing and MTT assay. For the study of γ -oryzanol effect on cholesterol uptake, the confluent cells were washed with HBSS twice and incubated with 1mL/well of micellar solutions containing 0.1mM cholesterol with or without 0.1mM γ -oryzanol for 2 hours at 37°C, followed by cell washing and cell lysis for Bradford protein assay and cholesterol analysis. Preincubation of cholesterol and γ -oryzanol in vials for 1, 2 or 6 hours at 37°C before being applied to cells for 2 hours was also studied.

4.2.6.2 Effect of γ -Oryzanol on Cholesteryl Oleate Hydrolysis

First of all, cholesteryl oleate hydrolysis facilitated by various concentrations of CEase was studied in test tubes by incubating 1mL of 0.1mM cholesteryl oleate and CEase in a serial of concentrations from 0, 10⁻⁵ to 1 μ g protein/mL at 37°C for 2 hours, since the CEase concentration should be limited and 2 hours incubation time was required for cholesterol uptake into cells. The

lowest concentration of CEase that resulted in significant cholesterol release from hydrolysis would be used to study the γ -oryzanol effect on CEase-facilitated cholesteryl oleate hydrolysis and consequently the availability of cholesterol in micelles, and the cholesterol uptake by human intestinal cells *in vitro*.

Secondly, cytotoxicity of 0.1mM cholesteryl oleate with or without CEase up to 10 μ g protein/mL (37°C, 2 hours) was studied to determine if the micellar solutions and the enzyme mixture would decrease cell viability. The cholesterol uptake into the cells was then studied, where the only source of cholesterol for uptake resulted from 0.1mM cholesteryl oleate hydrolysis (37°C, 2 hours) facilitated by various concentrations (0.001 to 10 μ g protein/mL) of CEase.

Thirdly, the γ -oryzanol effect on cholesteryl oleate hydrolysis and cholesterol, the product after hydrolysis, and uptake into cells was investigated by applying 0.1mM cholesteryl oleate with or without 0.1mM γ -oryzanol with 0.05 μ g protein/mL CEase to cells for 2 hours at 37°C. The γ -oryzanol effect on micellar solubility of cholesteryl oleate and consequently its availability for hydrolysis catalyzed by CEase was also studied by preincubating 0.1mM cholesteryl oleate micellar solutions with or without 0.1mM γ -oryzanol in vials for 1 hour at 37°C before being applied to cells for 2 hours at 37°C. After incubation, the cells were washed and lysed for Bradford protein assay and cholesterol analysis.

4.2.7 Solvent Extraction and Chemical Analysis

4.2.7.1 Analysis of γ -Oryzanol Using HPLC

To determine the γ -oryzanol concentration remaining in the micellar solutions, hexane/isopropanol (2:1:1) ($\times 1$) and hexane ($\times 3$) extraction was performed for each sample. The organic layers were collected, combined and evaporated under vacuum. The residue that

remained was dissolved in mobile phase and ultrasonicated to help dissolve if necessary, followed by HPLC.

A reverse-phase HPLC system developed by Xu and Godber (1999) was used to determine γ -oryzanol concentration. It consisted of a pump (Waters 510 HPLC Pump, Waters Co., Milford, MA), an autosampler (Waters 715 Sample Processor), a column (C_{18} , 25cm \times 4.6mm diameter, MICROSORB-MV 100 Å, Varian, Walnut Creek, CA) and a UV detector (wavelength 330 nm, Waters 486 Tunable Absorbance Detector). The mobile phase was methanol, acetonitrile, dichloromethane and acetic acid (50:44:3:3) with a flow rate maintained at 1.4 mL/min. One hundred μ L of each sample was injected each time. Retention times for γ -oryzanol was 5-9 minutes. Millenium³² software (Waters Co., Milford, MA) was used to record chromatograms and integrate peak areas. A standard curve of γ -oryzanol (concentration versus integrated area) was constructed to determine γ -oryzanol concentration in samples.

4.2.7.2 Cholesterol Analysis by GC/MS and Bradford Protein Assay

To determine cholesterol concentration in cell samples after treatment with test solutions, the cells were washed with HBSS twice and cell lysis was accomplished using 400 μ L/well of 1N NaOH at 37°C for 30 minutes, neutralized in 400 μ L/well of 1N HCl and pipetting up and down to break cell clumps to make a homogeneous cell suspension. Bradford protein assay was performed first to determine the amount of cell protein in the cell suspension. Two aliquots of 20 μ L homogenized cell suspension were diluted to 1mL with distilled deionized water and then mixed with 1mL of Bradford protein reagent. After 5 minutes at room temperature in the dark, the spectrum from 350 to 800nm was scanned first using a spectrophotometer to choose wavelengths for the maximum absorbance and the background. A serial dilution of bovine serum albumin at 0, 2, 4, 6 μ g/mL was used to construct a standard curve (concentration versus

absorbance ($A_{\text{max}} - A_{\text{background}}$). The crude protein concentration in each cell sample was calculated by converting the absorbance to concentration using the standard curve and multiplying the proper dilution factor.

The remaining cell suspension was spiked with 25 μ L of 1mM β -sitosterol in isopropanol as an internal standard and then extracted using hexane:isopropanol (3:1) ($\times 1$) and hexane ($\times 3$). A sample of cell suspension without being spiked with β -sitosterol after cells were incubated with 0.1mM γ -oryzanol micellar solution for 2 hours at 37°C and washed with HBSS twice was also tested to determine whether or not β -sitosterol existed. The organic layers were collected, combined and mixed with sodium sulfate anhydrous to remove water. The solvent was evaporated under vacuum. To determine cholesterol concentration, each sample with dried residue from cell suspension or micellar solution after γ -oryzanol analysis by HPLC was derivatized first with 100 μ L of trimethylsilylation reagents, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1), and 100 μ L pyridine to remove any possible water that could also react with the trimethylsilylation reagents. After incubation in a shaking water bath at 30°C for 2 hours, all samples were stored at 4°C until GC analysis, which was usually done within 24 hours.

The GC system (Varian CP-3800, Walnut Creek, CA) consisted of a glass injection port (splitless, 300°C) and a fused silica capillary column with low polarity (SPB-5, 30m x 0.25mm diameter and 0.1 μ m film thickness, Supelco Inc., Bellefonte, PA). The oven temperature was ramped from 100°C to 280°C with heating rate of 3°C/min and helium was the carrier gas maintained at a flow rate of 1.0mL/min. One μ L of each sample was injected with split ratio 0 from 0-0.2 minutes and 10000 from 0.2-60 minutes. The GC system was coupled with an MS detector (Varian Saturn 2200) with the following conditions: trap temperature 170°C, manifold

temperature 65°C and transfer line temperature 170°C; axial modulation 3.9 volts; mass range, m/z 45-650; EI auto ionization mode: scan time 0.39 seconds/scan, emission current 10μamps, pre-scan ionization time 100 μsecond and maximum ionization time 25000 μsecond.

The quantitation ions for area integration were 330+354+369+458 m/z for cholesterol and 397+358+382+487 m/z for β-sitosterol. The standard curves of cholesterol and β-sitosterol were constructed (concentration versus peak area). Total cholesterol concentration in each cell sample was normalized with β-sitosterol and expressed in μmole cholesterol/mg cell protein.

Statistical analysis used proc mix in SAS version 8.2 (SAS Institute Inc., Cary, NC) (see Appendix I for SAS codes).

4.3 Results and Discussion

4.3.1 Cholesterol Analysis by GC/MS and Internal Standard

After treatment, cells were usually lyzed in 1N NaOH or 1% sodium dodecyl sulfate (SDS) in order to prepare them for GC/MS analysis. Since lipid extraction would be performed later, NaOH was used instead of SDS, which could also be extracted and complicate the analysis. In this case, cholesteryl esters if present would be hydrolyzed to the free form of cholesterol in 1N of NaOH. Therefore, the cholesterol concentration measured was the total amount of cholesterol in cell suspension, including free and ester forms of cholesterol.

To determine the amount of cholesterol uptake into cells *in vitro*, cholesterol labeled with radioisotopes such as ^3H or ^{14}C and scintillation counter to measure radioactivity have typically been used. However, in this study GC/MS was used instead. There are several disadvantages to using radioisotopes including the safety issue and inconvenience such as that involving lipid extraction and other methods (i.e. thin layer chromatography) to separate the labeled compounds in the mixture and cleaning up waste, tools and machinery after use. On the other hand, GC/MS

provided a means to measure both free and ester forms of cholesterol or other substances simultaneously.

α -Cholestane has usually been used as an internal standard in cholesterol analysis, but in this case, because it had the same retention time as cholesteryl oleate under GC/MS analysis, it could interfere with the determination of certain ions selected for area integration in the calculation of concentration. β -Sitosterol was then used as an internal standard in this study because its chemical structure is similar to cholesterol. Also, β -sitosterol resulting from γ -oryzanol uptake into cells was not found in cell suspension determined by GC/MS and the CEase concentration used for cholesteryl ester hydrolysis was only 0.05 μ g protein/mL, which did not degrade γ -oryzanol and produce β -sitosterol (see the next chapter and Figure 5.7). Therefore, β -sitosterol concentration was not overestimated and it could be used as an internal standard in cell samples.

4.3.2 Effect of γ -Oryzanol on Micellar Solubility of Cholesterol and Cholesteryl Oleate

After 24-hour incubation of 0.1mM cholesterol or cholesteryl oleate micellar solution spiked with 0.1mM γ -oryzanol, cholesterol, cholesteryl oleate and γ -oryzanol all lost some degree of micellar solubility in this *in vitro* model. However, there were no significant differences after incubation with or without γ -oryzanol in cholesterol ($p=0.5622$) or in cholesteryl oleate ($p=0.3597$) recovered in the supernatant of the micellar solution (Figure 4.1(a) and (b) and Appendix III(a)). There was a tendency towards lower %recovery of cholesteryl oleate in the supernatant micelles than that of cholesterol, which indicated that γ -oryzanol might be more likely to affect cholesteryl oleate than cholesterol. The reasons may be related to differences in polarity and molecular weight. The polarity of cholesterol is higher with the free hydroxyl group than when it is esterified with a fatty acid. When forming a micelle in the

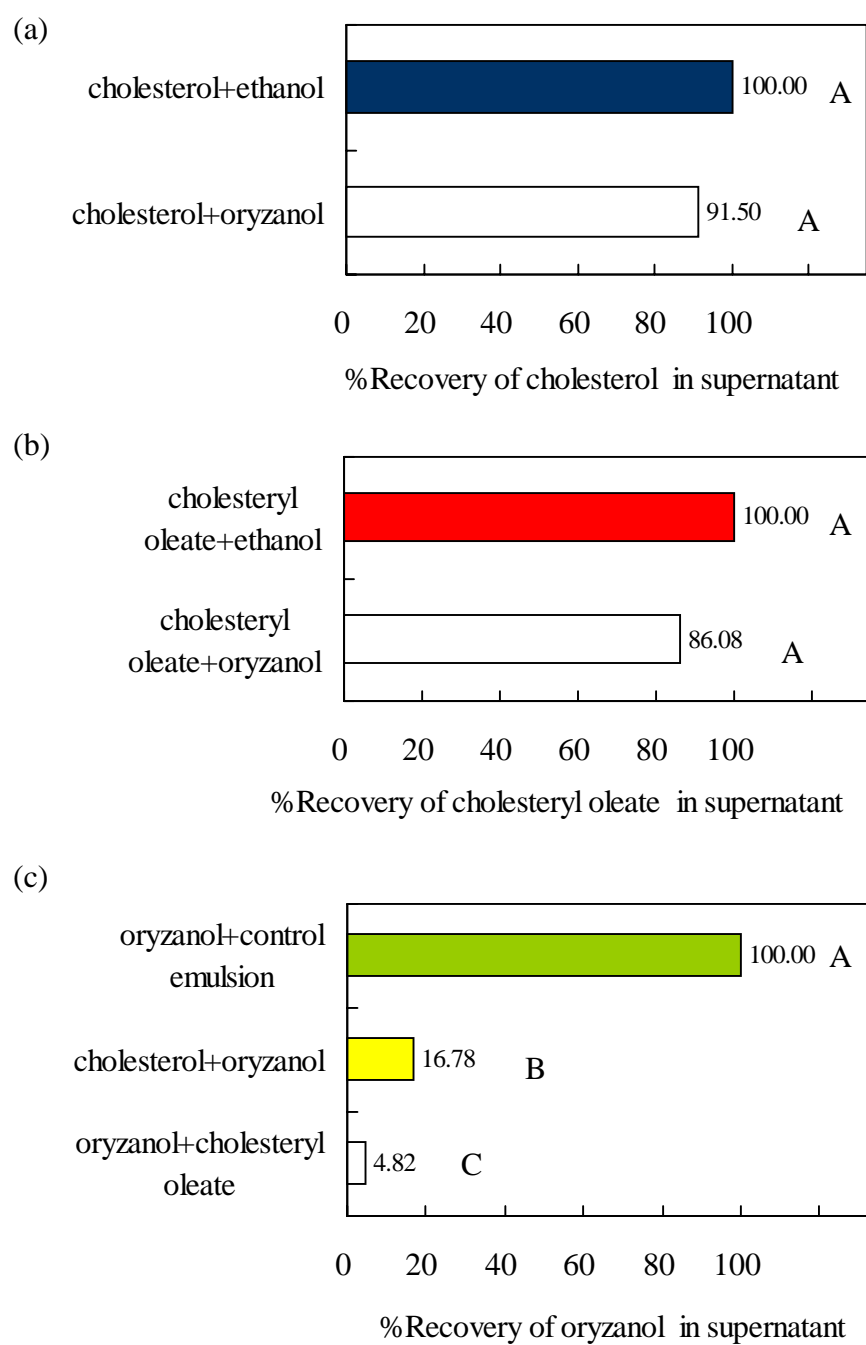


Figure 4.1. Effect of γ -oryzanol on micellar solubility of cholesterol and cholesteryl oleate. (n=2 \times 2 replications. Significant differences ($p<0.05$) are expressed by different letters.)

aqueous environment, the hydrophobic regions are in the interior while the hydrophilic regions are at the surface. As a larger molecule that is less polar, cholesteryl oleate would occupy more of the emulsifier's hydrophilic regions to remain stable in the micelle. With the fixed micellar composition, it is reasonable that the micellar solubility of cholesteryl oleate was affected more by γ -oryzanol, which is also a large molecule with low polarity.

Similarly, from the aspect of γ -oryzanol competing with cholesterol and cholesteryl oleate for the limited micellar space, %recovery of γ -oryzanol in the supernatant of micellar solution was significantly lower with the presence of cholesteryl oleate than that with cholesterol ($p < 0.05$) (Figure 4.1(c) and Appendix III(a)). This may also explain why micellar solubility of cholesteryl oleate was disturbed to a greater degree by γ -oryzanol.

Therefore, since hydrophobic substances such as cholesterol and cholesteryl oleate must form micelles in order for digestion and absorption in the human intestines, γ -oryzanol may have an effect to exclude cholesterol and cholesteryl oleate from the micelles and consequently may decrease the availability of cholesteryl oleate for hydrolysis and cholesterol for uptake into cells.

4.3.3 Effect of γ -Oryzanol on Cholesterol Esterase-Facilitated Cholesteryl Oleate Hydrolysis

Before the study of the γ -oryzanol effect, cholesteryl oleate hydrolysis facilitated by a serial concentration of CEase was investigated. The results showed that CEase was responsible for the release of cholesterol from cholesteryl oleate and the degree of cholesteryl oleate hydrolysis depended on the concentration of CEase (Figure 4.2). The higher the CEase concentration, the more cholesterol was released from cholesteryl oleate hydrolysis. In order to study the inhibitory effect of γ -oryzanol, the CEase concentration should be limited and much less than the substrate. Thus, CEase at concentration of 0.1 μg protein/mL was chosen because it

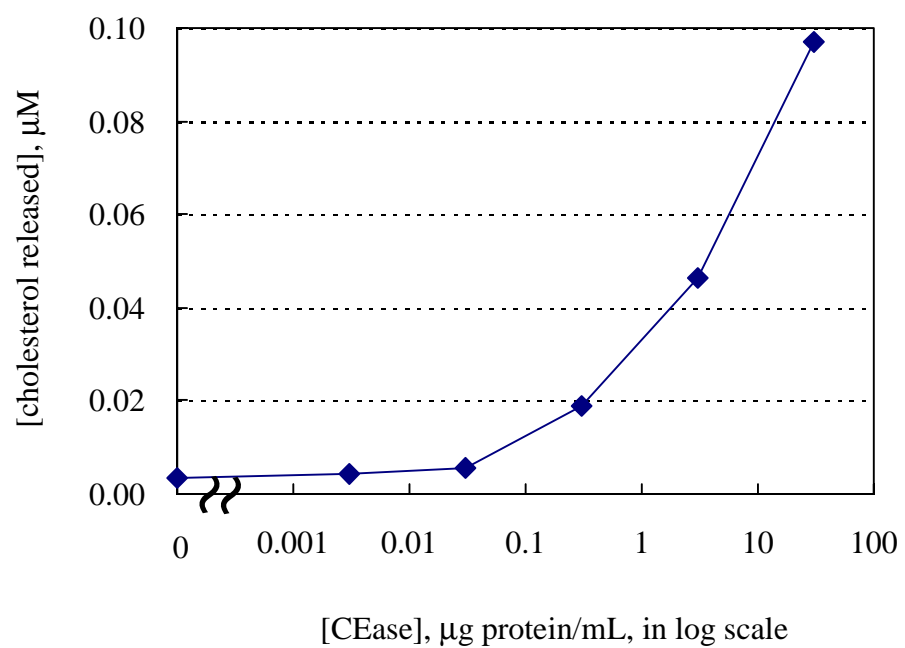


Figure 4.2. Cholesteryl oleate hydrolysis dependent on a serial concentration of cholesterol esterase, 37°C, 5 minutes

was the lowest concentration that showed significantly different activity from the control (no enzyme) ($p < 0.05$) in the production of detectable cholesterol.

A commonly used approach to studying enzyme mechanisms is through the use of the Michaelis-Menten equation, which was adapted in order to understand the inhibition effect of γ -oryzanol on CEase. One of the fundamental concepts in this approach is that the substrate concentration affects the rate of a reaction catalyzed by an enzyme. However, it is complicated to study substrate concentration effect since it changes over the reaction period. To simplify, the very short time following the start of the reaction, when the substrate concentration is much greater than the enzyme concentration, is studied instead. At the beginning, enzyme is mixed with the excess amount of substrate and the enzyme-substrate complex (ES) builds up quickly, which is called the pre-steady state and is usually too short to measure. Then a steady-rate state is achieved, in which the ES reaches equilibrium where the rate of ES breakdown is equal to ES formation. This kind of analysis is referred to as steady-state kinetics with the basic hypothesis by Michaelis and Menten that ES breakdown to free enzyme and product is the rate-limiting step in the reaction.

The initial reaction rate (or initial velocity), designated V_o , reflects the steady state and is determined by the ES breakdown to give product. In this case, V_o was measured as the rate of the product (cholesterol) concentration over the time of the early (first 5 minutes) reaction. The relationship between V_o and the substrate (cholesteryl oleate) concentration with a constant enzyme (CEase) concentration was constructed (Figure 4.3). It showed that if the cholesteryl oleate concentration reach infinity, V_o reaches constant at its maximum, designated V_{max} , and when V_o equals to a half of the V_{max} , the cholesteryl oleate concentration equals to Michaelis-Menten constant, designated K_m . The type of inhibition mechanism of γ -oryzanol could then be

determined by comparing K_{m1} and V_{max1} values without γ -oryzanol to K_{m2} and V_{max2} values with γ -oryzanol.

With these concepts, the study of the γ -oryzanol effect was done by incubating 0.2mM cholesteryl oleate with 0.1 μ g protein/mL CEase with or without 0.2mM γ -oryzanol at 37°C for 5 minutes. Even though V_o means initial rate in a sufficiently short time at the beginning of the reaction, incubation time less than 5 minutes was difficult to access and this approach was also used in the study of CEase activity by Mackay *et al.*, 1997. No internal standard was used in this case because β -sitosterol may be produced from γ -oryzanol degradation catalyzed by CEase and there was no proper internal standard in this case. The results expressed in Michaelis-Menten plot (Figure 4.3) showed that the effect of cholesteryl oleate concentration on cholesterol release was significant, which demonstrated the fundamental concept of this approach that the substrate concentration affects the rate of the reaction catalyzed by an enzyme.

By transforming a Michaelis-Menten plot to a double-reciprocal, or Lineweaver-Burk plot, $1/V_o$ (Y) versus $1/[\text{cholesteryl oleate}]$ (X) becomes a simple linear relationship ($Y=\beta_0+\beta_1X$), where the intercept (β_0) equals to $1/V_{max}$ and the slope (β_1) equals to K_m/V_{max} . The type of inhibition mechanism of γ -oryzanol could then be more easily observed (Figure 4.4) and compared by the changes of K_{m1} and V_{max1} of the straight line without γ -oryzanol to that, K_{m2} and V_{max2} , with γ -oryzanol.

Relative to γ -oryzanol inhibition, K_{m1} was not significantly different from K_{m2} and V_{max1} was also not significantly different from V_{max2} . Although not significant, the divergence in the plots might still suggest that γ -oryzanol tended to decrease V_{max} . This means that, with the presence of γ -oryzanol, reaction rate inclined to be lower possibly because γ -oryzanol may bind to CEase and inactivate it, thus reducing the concentration of active CEase and hence V_{max2}

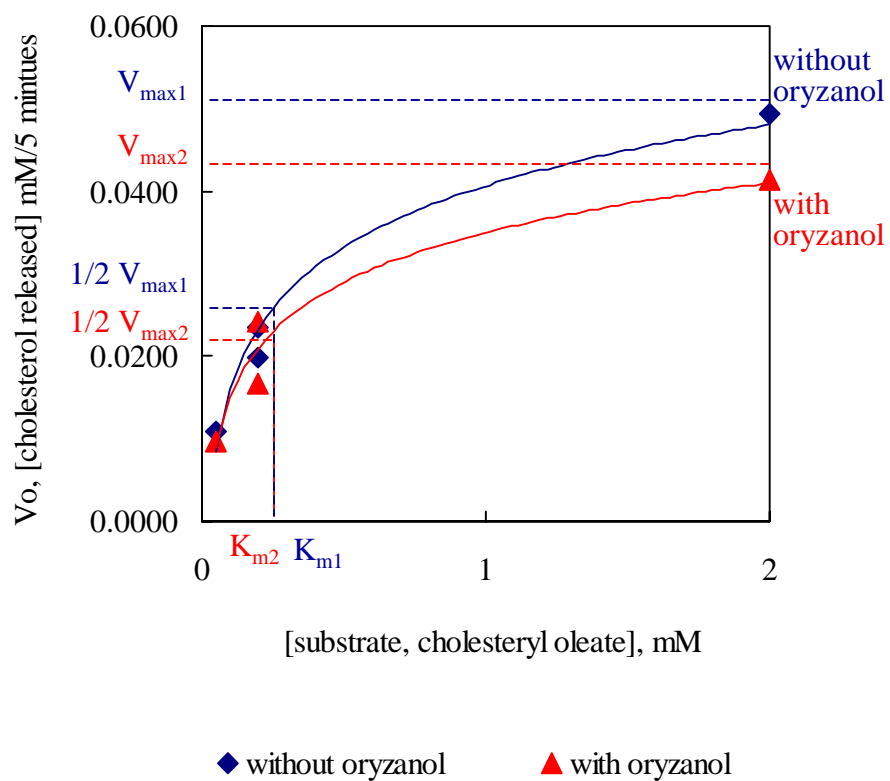


Figure 4.3. Michaelis-Menten plot of 0.2mM γ -oryzanol inhibition on 0.1 μ g protein/mL cholesterol esterase-facilitated cholesteryl oleate hydrolysis, 37°C, 5 minutes. (n=2 \times 2 replications)

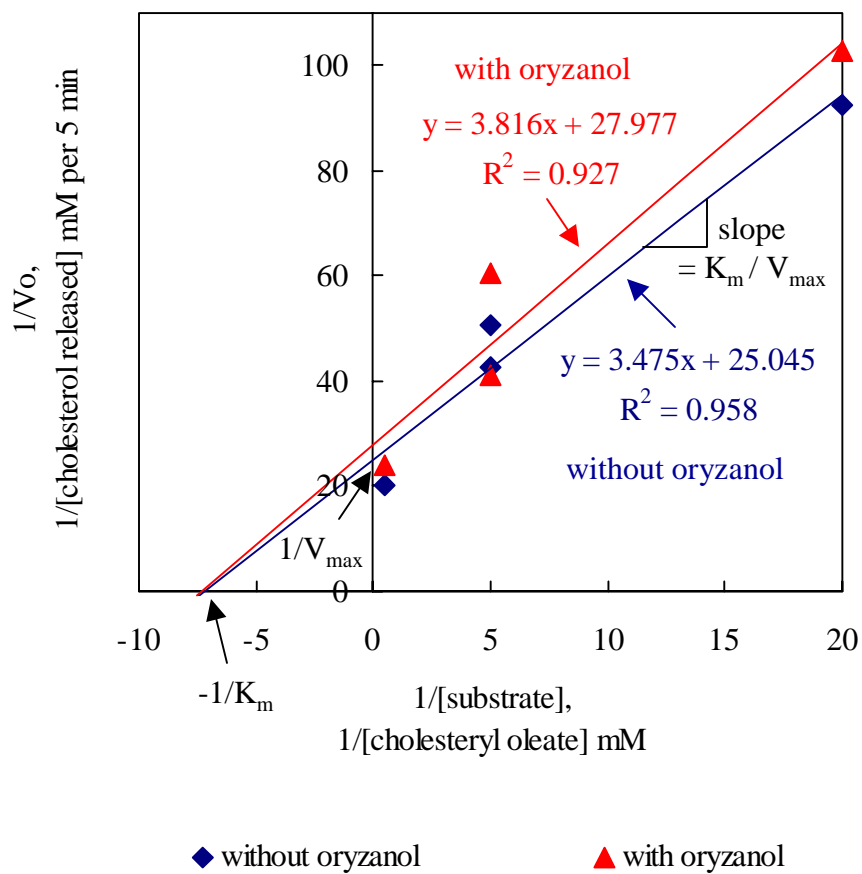


Figure 4.4. Lineweaver-Burk plot of 0.2mM γ -oryzanol inhibition on 0.1 μ g protein/mL cholesterol esterase-facilitated cholesteryl oleate hydrolysis, 37°C, 5 minutes. (n=2 \times 2 replications)

would decrease. However, the substrate concentration required (K_{m2}) to reach $1/2V_{max2}$ remained the same as that (K_{m1}) needed to reach $1/2V_{max1}$ without γ -oryzanol, which means that γ -oryzanol may bind to CEase at a site distinct from that which binds cholesteryl oleate. Thus, the inactivation occurred with or without the presence of cholesteryl oleate. This type of inhibition is referred to as noncompetitive inhibition. Therefore, it may be suggested that γ -oryzanol might have a tendency towards the inhibition of CEase and consequently the release of cholesterol from the hydrolysis was lowered. The reason may be the combined effects of γ -oryzanol on CEase inhibition and micellar solubility of cholesteryl oleate (less available for hydrolysis).

The mechanism of enzyme inhibition can be complicated, especially with measuring the initial velocity at the very beginning of the reaction. Also, it requires that the substrate concentration should be much higher than the enzyme concentration but it is unclear how high is acceptable. In addition, determination of the product concentration depends on the detection limit of the analytical method employed, which means the concentration of the product may be too low to be detected at the initial stage of the reaction. The greatest degree of CEase inhibition by γ -oryzanol may simply not be measured at the most appropriate moment. The use of an appropriate internal standard is also important if GC/MS is employed. More research is needed to further understand the characteristics of CEase, such as the hydrolytic steps from binding cholesteryl oleate to releasing cholesterol and the reaction rates, the active site of CEase responsible for cholesteryl ester hydrolysis and the γ -oryzanol binding site. Also, it must be determined if regulation of CEase by other substances is present and if the CEase inactivation will affect the digestion and metabolism of other compounds.

4.3.4 Cholesterol Uptake by Human Intestinal Cells *In Vitro*

In this study, the cell line used was C2BBel, a brush border expressing human colon cell line cloned from the Caco-2 cell line (ATCC HTB-37), which is derived from a human colon adenocarcinoma which has been used as a model in many studies of cholesterol uptake into the intestinal mucosa. Besides C2BBel, another cell line Fhs74Int (ATCC CCL-241), a human normal small intestine cell line, was also cultured with the intention to study cholesterol uptake *in vitro*. However, the cells required a relatively long time (2 weeks) to reach confluency and had limited passages before the onset of senescence. Therefore, the C2BBel cell line was developed to be a model for *in vitro* cholesterol uptake studies.

4.3.4.1 Effect of Cholesterol Esterase and Phospholipase A₂

The results of cytotoxicity studies (Figure 4.5) showed that 0.1mM cholesterol with or without CEase or phospholipase A₂ up to 100µg protein/mL at 37°C for 2 hours did not decrease cell viability in terms of cellular mitochondrial activity, compared to the control, serum-free and transferrin-free culture medium. For the effect of CEase and phospholipase A₂ on cholesterol uptake, the results (Figure 4.6) showed that neither enzyme up to 100µg protein/mL significantly increased cholesterol uptake into cells. Therefore, the study of the γ -oryzanol effect on cholesterol uptake could not address the possibility that γ -oryzanol could inhibit CEase and phospholipase A₂ activity. Rather the focus of this experiment became the effect of γ -oryzanol on the micellar solubility of cholesterol.

Initially, the study of the γ -oryzanol effect on CEase and phospholipase A₂ was motivated by the results from Mackay *et al.*, 1997, who suggested that CEase and phospholipase A₂ could promote cholesterol uptake by hydrolyzing phosphatidylcholine, one of the components used to prepare cholesterol in micelles, and therefore altering the physicochemical state of cholesterol in

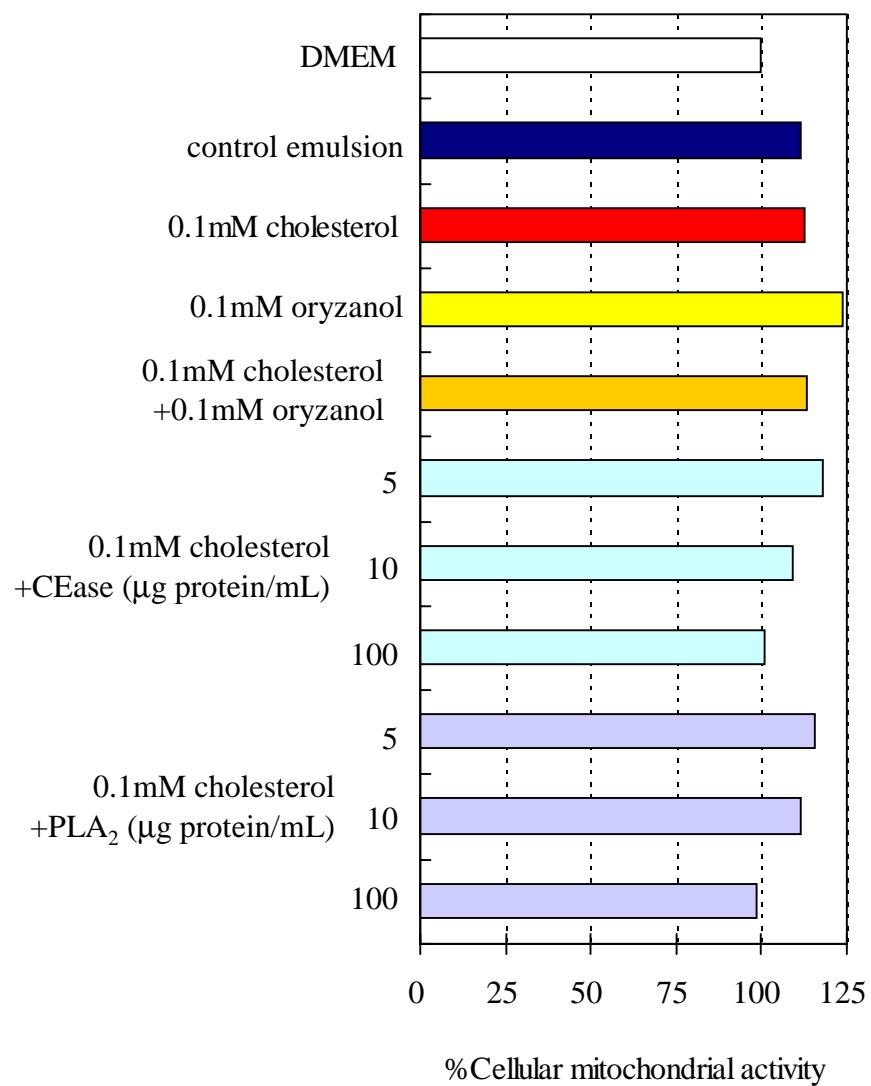


Figure 4.5. Cytotoxicity study of cholesterol incubated with γ -oryzanol, cholesterol esterase or phospholipase A₂ at 37°C for 2 hours

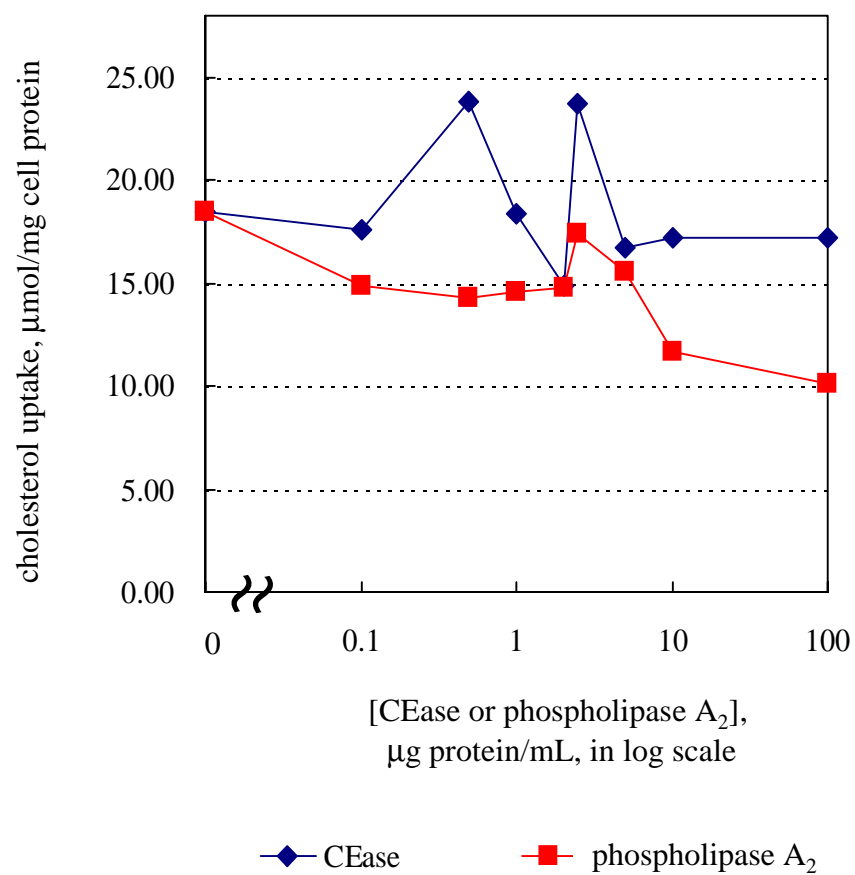


Figure 4.6. Effect of cholesterol esterase or phospholipase A_2 on cholesterol uptake into human intestinal cells *in vitro*, 37°C , 2hours. (n=2×3 replications)

the intestines. However, the same results were not observed in this compared with other studies (Shamir *et al.*, 1995, and Huang and Hui, 1990). One of the possible reasons is that all studies used different micellar compositions. The micelles used in this study to prepare cholesterol, cholesteryl oleate and γ -oryzanol in aqueous solution consisted of relatively high concentrations of oleic acid (0.5mM), 1-monolein (0.25mM) and phosphatidylcholine (0.3mM), since preparing γ -oryzanol micellar solution has been a challenge. Hence, micellar composition is critical in the investigation of micellar solubility of cholesterol or γ -oryzanol.

Moreover, while the molecule weight was not provided by the manufacturer, the concentrations of CEase and phospholipase A₂ could not be expressed in terms of molarity and be compared to the concentrations used in other studies. The concentrations of CEase and phospholipase A₂ in this study may not be sufficiently high enough to promote cholesterol uptake. In summary, the lipid composition of micelles in human intestines is very complicated and therefore, more research is needed to further understand the roles of CEase and phospholipase A₂ in promoting cholesterol uptake.

4.3.4.2 Effect of γ -Oryzanol on Availability of Cholesterol for Uptake

First of all, the results of cytotoxicity studies showed that 0.1mM cholesterol with or without 0.1mM γ -oryzanol did not decrease cellular mitochondrial activity compared to the control, serum-free and transferrin-free culture medium (Figure 4.5). As for the cholesterol uptake, the concentration of cholesterol recovered from the cell suspension was significantly higher in cells incubated with cholesterol than that in the control emulsion without cholesterol ($p < 0.05$) (Figure 4.7 and Appendix III(b)). Since cholesterol is one of the natural constituents in the cell membrane of animal cells, even the control sample should test positive for cholesterol

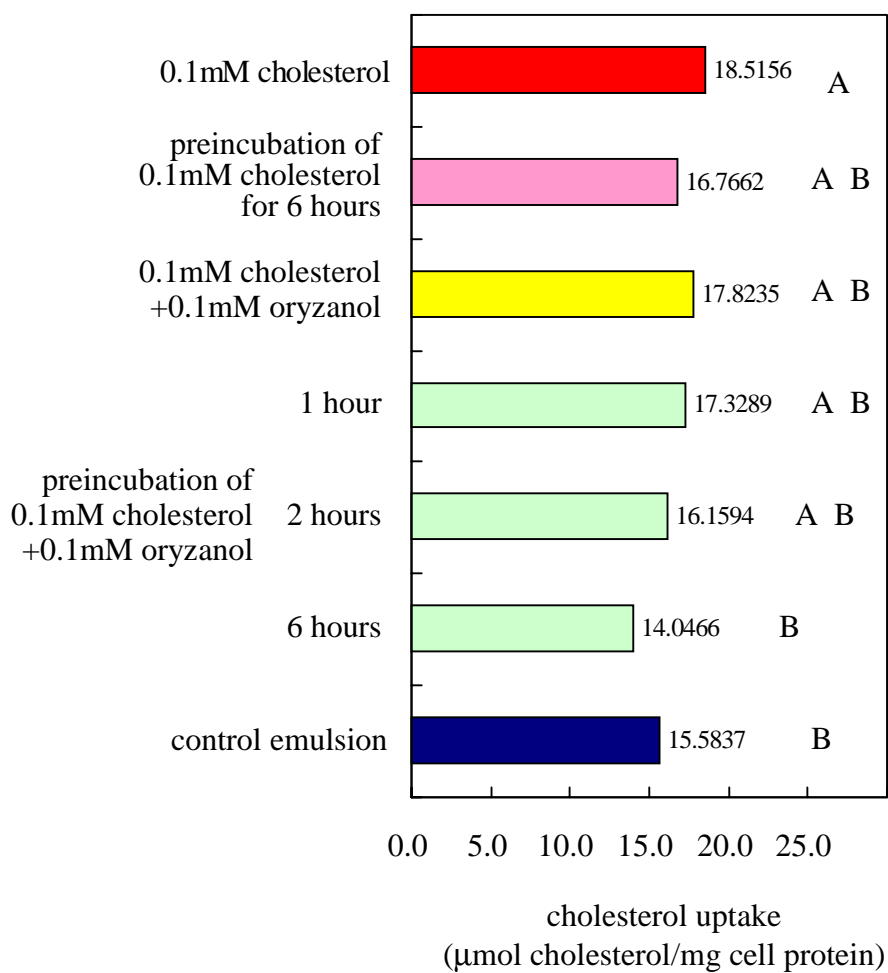


Figure 4.7. Effect of γ -oryzanol preincubated with cholesterol for 0, 1, 2 or 6 hours on cholesterol uptake into human intestinal cells *in vitro*, 2 hour, 37°C. (n=2×3 replications. Significant differences ($p<0.05$) are expressed by different letters.)

and the extra cholesterol must have resulted from the cholesterol uptake into cells or association with cells.

The results also showed that, with or without the presence of γ -oryzanol, the cholesterol concentrations recovered were not significantly different ($p=0.6808$). When cholesterol and γ -oryzanol were preincubated in vials for up to 6 hours before being applied to cells, the concentrations of cholesterol recovered from cell suspensions tended to decrease more obviously in a time-dependent manner. The longer the preincubation, the less cholesterol recovered. Preincubation time for two hours did not show a significant difference ($p=0.2105$) but it showed a significant decrease after six-hour preincubation ($p<0.05$) to a level equivalent to the control. The preincubation of cholesterol alone for 6 hours caused less cholesterol uptake than without preincubation but not significantly. Therefore, preincubation with γ -oryzanol for 6 hours had a significant effect on decreasing cholesterol uptake and it may be due to the decreased cholesterol micellar solubility in preincubation.

4.3.4.3 Effect of γ -Oryzanol on Cholesteryl Oleate Hydrolysis

The results of the relationship between various concentrations of CEase and the cholesterol released from hydrolysis is shown in Figure 4.8. The relationship was concentration-dependent, which means that the more CEase catalyzed the hydrolysis, the more cholesterol was produced. The concentration range studied was only up to $1\mu\text{g}$ protein/mL, which resulted in only about 5% cholesterol production. It was postulated that, when applied to cells, the reaction direction would go in favor of releasing products, which would be taken up into cells, and the hydrolysis may not reach equilibrium. Therefore, CEase at concentration as low as $0.05\mu\text{g}$ protein/mL was significantly different from the control (no enzyme) ($p<0.05$) and was sufficient to be used later to study γ -oryzanol effect on cholesteryl oleate hydrolysis.

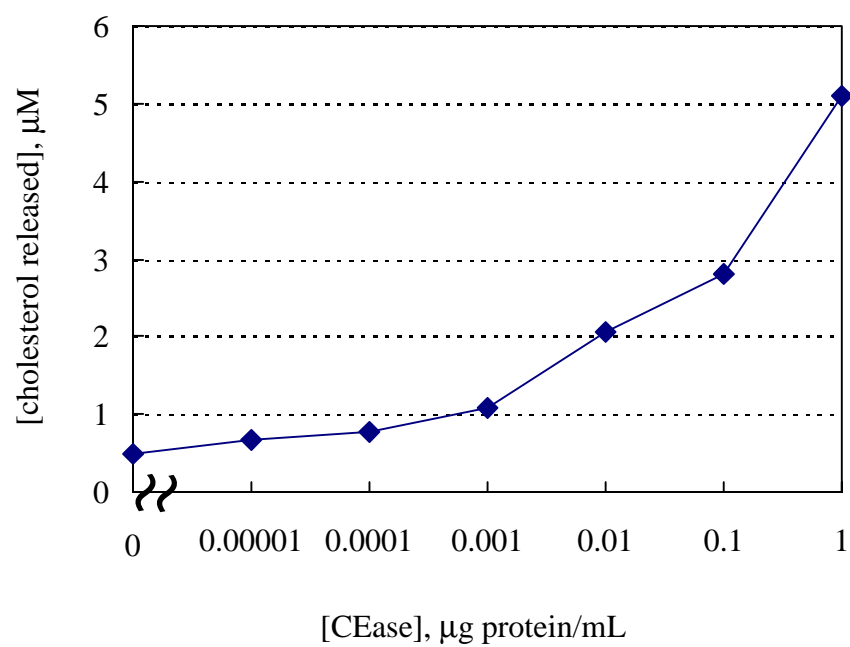


Figure 4.8. Cholesterol esterase concentration effect on cholesteryl oleate hydrolysis, 37°C, 2 hours

As for the cytotoxicity studies, the results showed that 0.1mM cholesteryl oleate with or without CEase up to 10 μ g protein/mL did not decrease cellular mitochondrial activity compared to the control, serum-free and transferrin-free culture medium (Figure 4.9). For the uptake studies, the results showed that, after cholesteryl oleate was incubated with cells, there was no significant cholesterol uptake compared to the control emulsion (Figure 4.10 and Appendix III(c)). The results also agreed with Mackay *et al.*, 1997, that the ester form of cholesterol could not be absorbed without prior hydrolysis. The dietary cholesteryl esters are usually hydrolyzed by pancreatic CEase to free cholesterol in intestines before uptake. It also showed that when cholesteryl oleate was incubated with 0.05 μ g protein/mL CEase, there was significantly higher cholesterol concentration detected in the cell suspension ($p<0.05$). Thus, the results suggested that cholesteryl esters could not be absorbed by intestinal cells, and in order for uptake, hydrolysis catalyzed by CEase was necessary. The study of γ -oryzanol effect would then focus on inhibition on CEase hydrolytic activity and micellar solubility of cholesteryl oleate (availability for hydrolysis).

First of all, the cytotoxicity results showed that γ -oryzanol incubated with cholesteryl oleate and CEase did not decrease the mitochondrial activity of cells (Figure 4.9). Secondly, with the presence of γ -oryzanol, the uptake of cholesterol resulted in a trend towards decreased cholesteryl oleate hydrolysis ($p=0.1392$) (Figure 4.10 and Appendix III(c)). The reason may be due to the combined effects of γ -oryzanol on micellar solubility of cholesteryl oleate and cholesterol, and on CEase inhibition. It may be suggested that when γ -oryzanol and cholesteryl oleate are simultaneously present in the intestines, γ -oryzanol can affect the cholesteryl oleate hydrolysis and then decrease the cholesterol uptake into cells.

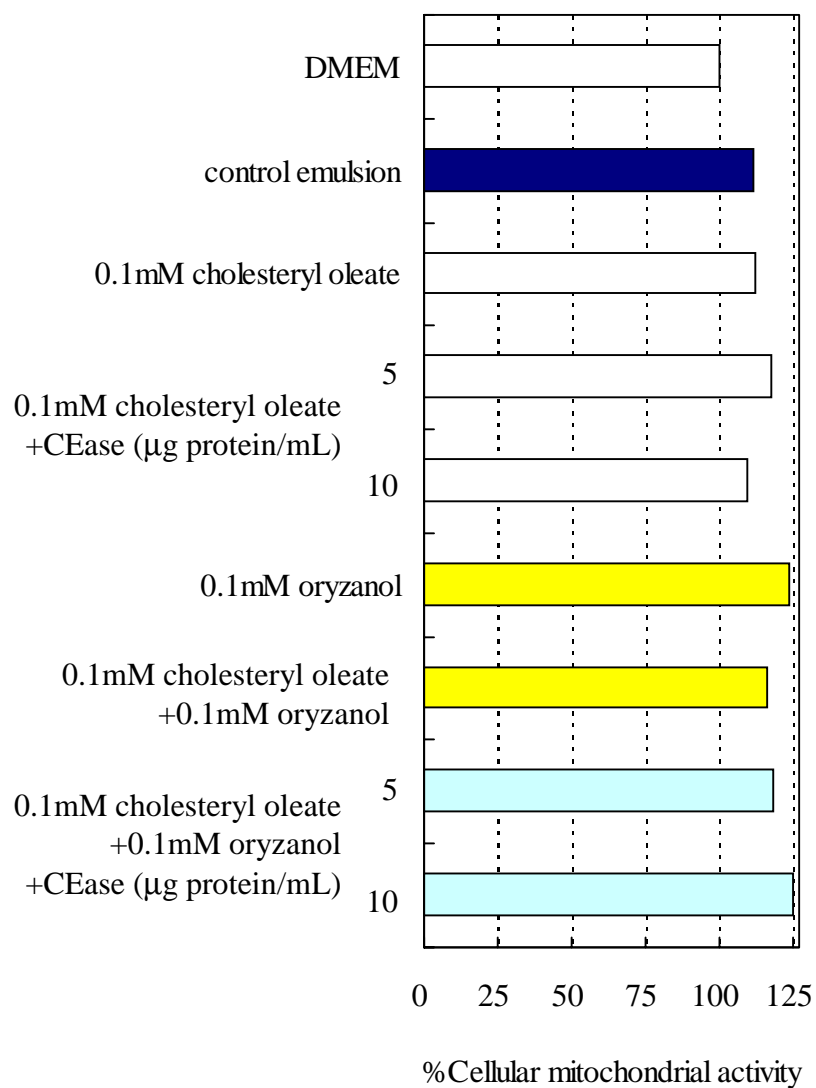


Figure 4.9. Cytotoxicity study of cholesteryl oleate incubated with γ -oryzanol and cholesterol esterase at 37°C for 2 hours

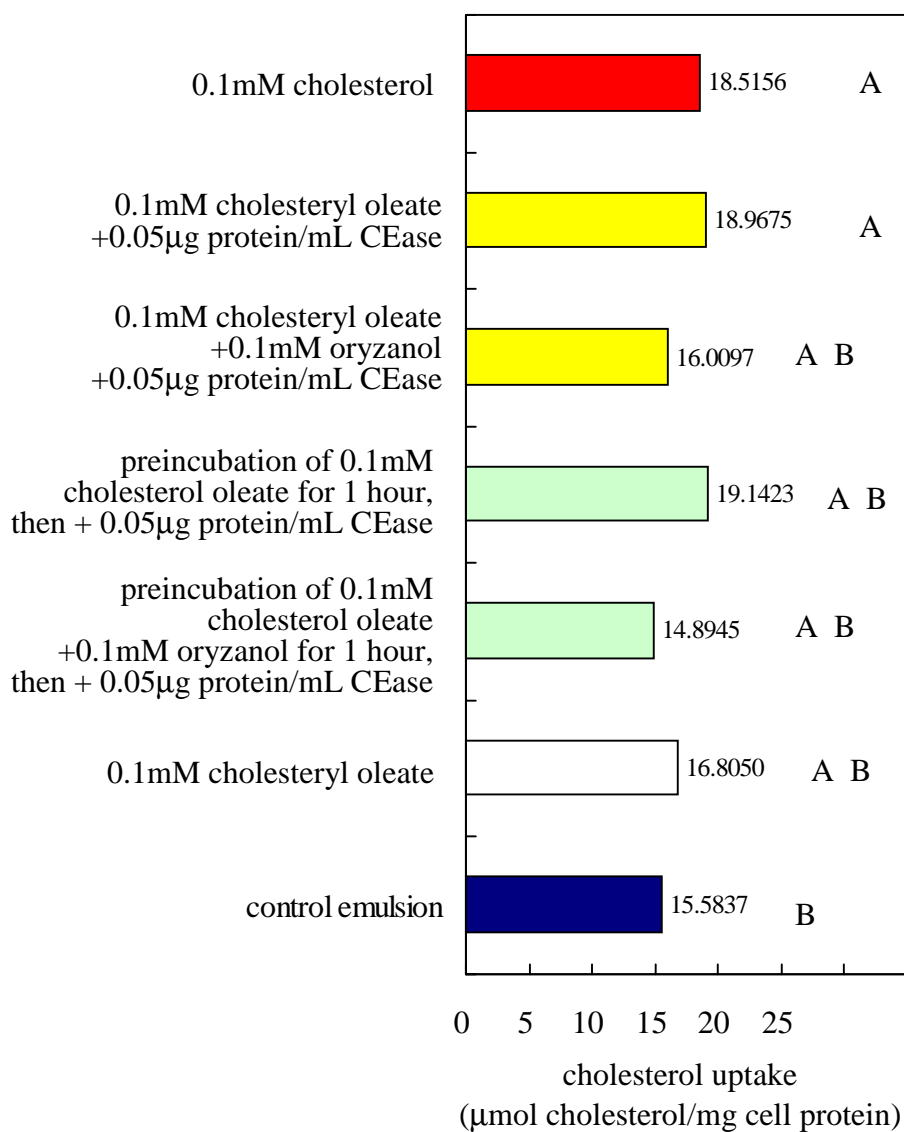


Figure 4.10. Effect of γ -oryzanol preincubated with cholesteryl oleate for 0 or 1 hour before cholesterol esterase-facilitated hydrolysis on cholesterol uptake into human intestinal cells *in vitro*, 2 hour, 37°C. (n=2×3 replications. Significant differences ($p < 0.05$) are expressed by different letters.)

Furthermore, after preincubation with γ -oryzanol in vials at 37°C for 1 hour, cholesteryl oleate hydrolysis by 0.05 μ g protein/mL CEase resulted in a trend towards lower cholesterol uptake ($p=0.1546$) (Figure 4.10 and Appendix III(c)). This may be because γ -oryzanol disturbed the micellar solubility of cholesteryl oleate or decreased the availability of cholesteryl oleate for hydrolysis and cholesterol production, and therefore decreased cholesterol uptake. Thus, it may be suggested that if γ -oryzanol is present with cholesteryl oleate for more than one hour before interacting with CEase in the intestines, the availability of cholesteryl esters may decrease further due to competition with γ -oryzanol and by being excluded from micelles. After interacting with CEase, hydrolysis of cholesteryl esters might be inhibited by γ -oryzanol resulting in less free form of cholesterol available for uptake and also the micellar solubility of the hydrolysis product, cholesterol, may decrease, thus the cholesterol uptake into cells may consequently decrease.

Additionally, differences occurred in the preincubation times required for γ -oryzanol to reduce cholesterol uptake between free cholesterol and cholesteryl ester. Preincubation of γ -oryzanol with cholesteryl oleate for 1 hour before being introduced to cells showed an effect on cholesterol uptake relatively greater than preincubation with free cholesterol. The reason may be due to their chemical structures and the stability of micelles as stated in Section 4.3.2. Free cholesterol is more hydrophilic than its ester form and, reasonably, to decrease its micellar solubility may take a longer time than with the esters. Therefore, it suggested that, in the gastrointestinal tract before uptake, γ -oryzanol might play a bigger role in affecting micellar solubility of cholesteryl esters than in affecting the free form of cholesterol.

Finally, when enzymes are involved, the mechanism of inhibition can be complicated. For example, the hydrolysis and uptake of cholesterol, which was the product from the hydrolysis, most likely occur simultaneously. Consequently, the reaction would not reach

equilibrium because the product concentration would not increase over time (due to uptake into cells) and the reaction direction would go toward the product. Even a small amount of CEase may be able to facilitate hydrolysis over time as was the case with the CEase at the higher concentration in this study. Hence, the γ -oryzanol effect on CEase inhibition might be difficult to detect. Thus, the focus of this study became the γ -oryzanol effect on micellar solubility of cholesteryl oleate, or availability of cholesteryl oleate for hydrolysis.

4.4 Conclusion

Many researchers have studied and found that γ -oryzanol possesses hypocholesterolemic capacity. However, this is the first time that the possible mechanism of this γ -oryzanol function has been approached. In addition, a cell model using human intestinal cells accompanied with GC/MS was developed and found capable of detecting cholesterol uptake into cells *in vitro*.

The mechanism of the hypocholesterolemic capacity of γ -oryzanol is complicated. Since the results of digestibility and absorbability of γ -oryzanol suggested that γ -oryzanol is poorly absorbed by intestinal cells (see next chapter), therefore, γ -oryzanol may affect cholesterol uptake as it takes place in the lumen of the gastrointestinal tract. This study tried to distinguish the avenues that γ -oryzanol may influence and then develop a model to investigate the γ -oryzanol effect from each aspect.

From the results, the hypocholesterolemic capacity of γ -oryzanol could be envisioned from three standpoints. 1) Because the uptake of cholesteryl oleate was not found but the hydrolysis catalyzed by CEase that generated free cholesterol was, γ -oryzanol may affect the availability of cholesteryl esters for CEase-facilitated hydrolysis by reducing the micellar solubility of cholesteryl esters. 2) Since dietary cholesteryl esters are usually hydrolyzed by pancreatic CEase in the small intestines, and CEase was not found to promote free cholesterol

uptake, the effect of γ -oryzanol focused on the inhibition of hydrolytic function of CEase, which was to inhibit the hydrolysis of cholesteryl esters and reduce free cholesterol production. 3) Because it was not found that the uptake of free cholesterol was promoted by CEase and phospholipase A₂, γ -oryzanol may affect the availability of free cholesterol for uptake by decreasing its micellar solubility. Therefore, consequently, the cholesterol uptake into cells decreased.

Some researchers also studied the binding of bile salts (i.e. Nagaoka *et al.*, 1999, and Ikeda *et al.*, 1992) as a mechanism for serum cholesterol reduction. Bile salts are excreted by the liver and stored in and released from gallbladder to duodenum in response to digestion. They are amphipathic with both hydrophilic and hydrophobic regions and function as emulsifiers to prevent gallstone formation resulting from the precipitation of cholesterol in bile and to form micelles containing dietary lipids. Hence, it is reasonable that researchers should also consider the study of the effects of γ -oryzanol on binding bile salts since the reduction of bile salt availability for micelle formation in the small intestines would result in a decrease of micellar solubility of cholesterol along with other lipids.

In order to do so, what must be kept in mind, however, is the analytical method to measure bile salts, i.e. enzymatic assay using hydroxysteroid dehydrogenase should have specificity to bile salts only and not react with γ -oryzanol to give false positives. This is because, unlike the soy protein peptic hydrolysate in the study by Nagaoka *et al.*, 1999, and tea catechins in Ikeda *et al.*, 1992, oryzanol and bile salts have similar ring structures.

CHAPTER 5. *IN VITRO* DIGESTIBILITY AND ABSORBABILITY OF γ -ORYZANOL

5.1 Introduction

The absorption and metabolism of γ -oryzanol in human or animal bodies has not been fully delineated. There are only a few of studies in English exploring this phenomenon. Many of the studies done in the area have used animal models such as rabbits and rats (Fujiwara *et al.*, 1983). Measurement of γ -oryzanol and its metabolites in those studies employed thin layer chromatography, which was the only method available prior to the development of modern chromatographic approaches to analysis.

Therefore, with the assistance of liquid chromatography, gas chromatography coupled with mass spectrometry and cell culture techniques, the objective of this study was to investigate the metabolic fate of γ -oryzanol in the gastrointestinal tract by an *in vitro* model involving simulated peptic and pancreatic digestion and by cell culture using human intestinal cells. The effect of three specific pancreatic enzymes, cholesterol esterase (CEase), lipase and phospholipase A₂, on γ -oryzanol degradation and the identification of the degradation products were studied.

5.2 Materials and Methods

5.2.1 Materials and Reagents

γ -Oryzanol was obtained from crude rice bran oil using preparative normal-phase high performance liquid chromatography (HPLC) and crude rice bran oil was extracted from milled rice bran according to the method developed by Xu and Godber (1999). The preparative HPLC system consisted of a Waters (Milford, MA) Prepak[®] RCM base connected with two cartridges (each was a 25mm×10cm Prep Nova-Pak HR silica with particle size 6 μ m) and a Guard-Pak insert, a U6K manual injector, a 510 pump and a Lambda-Max 481 LC Spectrophotometer

(wavelength 330 nm). Mobile phase was methanol with a constant flow rate at 15 mL/min and 2mL of the crude oil were injected each time. The eluate containing γ -oryzanol was collected (25-30min) and the solvent was evaporated.

Oleic acid, 1-monoolein, lecithin, sodium taurocholate, ferulic acid, stigmasterol, Na_2HPO_3 , MTT assay kit, Bradford reagent, Hank's balanced salts, porcine bile extract, porcine pepsin, porcine pancreatin, containing many enzymes including amylase, trypsin, lipase, ribonuclease and protease, porcine pancreatic cholesterol esterase, lipase, phospholipase A_2 and trimethylsilylation reagents (N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1)) were purchased from Sigma, St. Louis, MO. HCl and NaHCO_3 were purchased from Mallinckrodt, Inc., Paris, KY. NaOH, NaCl, KH_2PO_3 , hexane and isopropanol were purchased from Fisher Scientific, Fair Lawn, NJ. Tris(hydroxymethyl)aminomethane was purchased from Amresco, Solon, OH. Culture plates were purchased from Corning Inc., Corning, NY, and the sterile disposable syringe filter was 0.2 μm PES purchased from Whatman, Clifton, NJ.

Phosphate buffered saline (PBS) was prepared by dissolving 0.9g of NaCl, 0.111g of Na_2HPO_3 and 0.03g of KH_2PO_3 in about 90mL of distilled deionized water, adjusting pH to 7 with 10N NaOH and diluting to 100mL volume with water. Tris-HCl buffer (0.1M) was prepared by dissolving 1.2114g of Tris(hydroxymethyl)aminomethane in about 90mL of distilled deionized water, adjusting pH to 7.5 with 10N of HCl and diluting to 100mL volume with water.

5.2.2 Preparation of γ -Oryzanol Micellar Solution

γ -Oryzanol micellar solution was prepared by first dissolving 0.1mM γ -oryzanol mixed with 0.5mM oleic acid, 0.25mM 1-monoolein and 0.3mM lecithin in solvent (hexane:isopropanol=3:1). After the solvent was dried under a vacuum evaporator with temperature set at 45°C (CentriVap Console, Labconco Inc., Kansas City, MO), 6.6mM sodium

taurocholate in buffer or culture medium free of serum and transferrin was added to the dried lipids and then ultrasonicated (Sonic Dismembrator 60, Fisher Scientific, Fair Lawn, NJ) in an ice bath for 45 minutes. The micellar solution was free of precipitate.

5.2.3 Preliminary Study of *In Vitro* Digestibility of γ -Oryzanol

Digestibility of γ -oryzanol was determined by a modified *in vitro* model (Ames *et al.*, 1999) to simulate digestion in the human stomach and small intestines. Crystalline γ -oryzanol, 1mg, or 500 μ L of 0.2mM γ -oryzanol micellar solution in PBS (1mM oleic acid, 0.5mM 1-monoolein and 0.6mM lecithin, without sodium taurocholate) was mixed with 3mL of distilled water and 2mL of freshly made pepsin solution (0.32g/mL in 0.1N HCl) and pH was adjusted to 2 using 6N HCl. After peptic digestion in a shaking water bath at 37°C for 2 hours, 1mL of a freshly prepared bile (25mg/mL) and pancreatin (4mg/mL) mixture in 0.1N NaHCO₃ was added to the digest with pH adjusted to 7 using 0.5N NaHCO₃ and incubated at 37°C for 2 hours. Digestibility of ferulic acid and stigmasterol (1.0mg crystalline each) were also tested.

5.2.4 Digestibility of γ -Oryzanol *In Vitro* by Pancreatic Enzymes, Cholesteryl Esterase, Lipase and Phospholipase A₂

To investigate the effect of specific pancreatic enzymes on digestibility of γ -oryzanol, CEase, lipase and phospholipase A₂ were studied. Each enzyme at a serial concentrations of 0.1, 1, 10, 100 or 500 μ g protein/mL was applied to 1mL of 0.1mM γ -oryzanol micellar solution containing 0.5mM oleic acid, 0.25mM 1-monoolein, 0.3mM lecithin and 6.6mM sodium taurocholate in 0.1mM Tris-HCl buffer (pH 7.5) for 1.5 hour at 37°C, followed by solvent extraction, determination of the remaining γ -oryzanol concentrations by HPLC analysis and identification of digestion products by a gas chromatography (GC) coupled with mass spectrometry (MS) analysis.

5.2.5 Study of γ -Oryzanol Uptake by Human Intestinal Cells *In Vitro*

5.2.5.1 Cell Culture

γ -Oryzanol uptake by human intestines *in vitro* was investigated by using C2BBel cells (ATCC CRL-2102). To maintain the cell line, the cells were subcultured every 4 days when confluent. The old medium was pipetted out and discarded and the cells were washed with 0.25% trypsin solution three times. At the third time the trypsin solution remained until the cells detached from the flask (about 10 minutes at 37°C). The cell suspension was pipetted up and down to break any clumps and subcultured (split ratio 1:3) with new medium (90% DMEM with 4mM L-glutamine, 1.5g/L sodium bicarbonate and 4.5g/L glucose and supplemented with 0.01mg/mL human transferrin; 10% fetal bovine serum). For γ -oryzanol uptake experiments, the cell suspension was diluted in new medium to 2×10^5 cells/mL and 1mL was transferred to each well (area=2cm²) of the 24-well culture plates, which resulted in 10⁵cells/cm². For cytotoxicity studies, 96-well plates were used and 100 μ L of the cell suspension was cultured in each well. Cell count was performed at each subculture to ensure consistency for each experiment.

5.2.5.2 Cytotoxicity Study of γ -Oryzanol Micellar Solution Using an MTT Assay

To study cytotoxicity of γ -oryzanol micellar solution, a 3-(4,5-dimethylthiazol-yl)-2-5-diphenyltetrazolium bromide (MTT) assay kit was used to measure cell viability in terms of the activity of mitochondrial dehydrogenases in living cells. After subculture in 96-well culture plates and reaching confluency, the cells were washed gently twice with Hank's balanced salt solution (HBSS) and applied at 100 μ L/well of γ -oryzanol micellar solution prepared in culture medium free of serum and transferrin for 2 hours at 37°C. After incubation, the γ -oryzanol solution was removed and the cells were gently washed twice with HBSS, followed by 40 μ L/well of 1.25mg/mL MTT in HBSS, which has a yellow color. After incubation at 37°C in

the dark for 2 hours, the mitochondrial dehydrogenases of viable cells cleaved the tetrazolium ring and yielded purple formazan crystals, which are insoluble in aqueous solution. Then 100 μ L/well of MTT solubilization solution containing 10% Triton X-100 plus 0.1N HCl in anhydrous isopropanol was added to solubilize purple formazan crystals. Pipetting up and down at least 100 times helped solubilization in dense culture situation. Absorbances at 570nm for color development and at 690nm for background were read using a microplate spectrophotometer (SPECTRAmax® PLUS, Molecular Devices Co., Sunnyvale, CA). An increase or decrease of cell numbers after exposure to a test solution would correspond to a concomitant change in the amount of purple formazan formed or in the optical density at absorbance 570nm. The cell viability under exposure to a test compound was expressed in %cellular mitochondrial activity, which was the ratio of absorbance reading difference between 570nm and 690nm of test compound ($A_{570} - A_{690}$) to that of the control ($A_{570}^0 - A_{690}^0$):

$$\% \text{Cellular mitochondrial activity} = \frac{A_{570} - A_{690}}{A_{570}^0 - A_{690}^0} \times 100\%$$

5.2.5.3 Study of γ -Oryzanol Uptake by Cells *In Vitro*

C2BBe1 cells were incubated with 1mL/well of the γ -oryzanol micellar solution in serum-free and transferrin-free culture medium for 2 hours at 37°C. After incubation, the test solution was pipetted out and the cells were washed gently with HBSS twice. The cells were lysed in 400 μ L/well of 1N NaOH at 37°C for 30 minutes and neutralized by 400 μ L/well of 1N HCl before further γ -oryzanol determination.

5.2.6 Analysis of γ -Oryzanol and Ferulic Acid Using Solvent Extraction and HPLC

To determine the remaining γ -oryzanol and ferulic acid concentrations and the hydrophobic digestion products after digestion, hexane/ethyl acetate/isopropanol (2:1:1) ($\times 1$) and

hexane (×3) extraction was performed for each sample. The organic layers were collected, combined and mixed with sodium sulfate anhydrous to remove water. The solvent was evaporated under vacuum. The residue remaining was dissolved in mobile phase and ultrasonicated to help dissolve if necessary, followed by liquid chromatography to determine concentrations of γ -oryzanol and ferulic acid.

A reverse-phase HPLC system developed by Xu and Godber (1999) was used to determine γ -oryzanol and ferulic acid concentrations. It consisted of a pump (Waters 510 HPLC Pump, Waters Co., Milford, MA), an autosampler (Waters 715 Sample Processor), a column (C_{18} , 25cm×4.6mm diameter, MICROSORB-MV 100 Å, Varian, Walnut Creek, CA) and a UV detector (wavelength 330 nm, Waters 486 Tunable Absorbance Detector). The mobile phase was methanol, acetonitrile, dichloromethane and acetic acid (50:44:3:3) with a flow rate maintained at 1.4 mL/min. One hundred μ L of each sample was injected each time. Retention times for γ -oryzanol and ferulic acid were 5-9 minutes and 1.5 minutes, respectively. Millenium³² software (Waters Co., Milford, MA) was used to record the chromatograms and integrate peak areas. A standard curve of γ -oryzanol (concentration versus integrated area) was constructed to infer γ -oryzanol concentration in samples.

5.2.7 Identification of γ -Oryzanol Digest Products by GC/MS

After HPLC analysis, the solvent in each remaining sample was evaporated for further gas chromatography and mass spectrometry (GC/MS) analysis. Before being injected into GC/MS, each sample with dried residue was derivatized using 100 μ L of trimethylsilylation reagents, which were N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1), and 100 μ L pyridine to remove any possible water, which could also react with the trimethylsilylation reagents. After incubation in a shaking water bath at

30°C for 2 hours, all samples were stored at 4°C until GC analysis, which was usually done within 24 hours.

The GC system (Varian CP-3800, Walnut Creek, CA) consisted of a glass injection port (splitless, 300°C) and a fused silica capillary column with low polarity (SPB-5, 30m x 0.25mm diameter and 0.1µm film thickness, Supelco Inc., Bellefonte, PA). The oven temperature was ramped from 100°C to 280°C with heating rate of 3°C/min and helium was the carrier gas maintained at a flow rate of 1.0mL/min. One µL of each sample was injected with split ratio 0 from 0-0.2 minutes and 10000 from 0.2-60 minutes. The GC system was coupled with an MS detector (Varian Saturn 2200) with the following conditions: trap temperature 170°C, manifold temperature 65°C and transfer line temperature 170°C; axial modulation 3.9 volts; mass range, m/z 45-650; EI auto ionization mode: scan time 0.39 seconds/scan, emission current 10µamps, pre-scan ionization time 100 µsecond and maximum ionization time 25000 µsecond.

5.3 Results and Discussion

5.3.1 Digestibility of γ -Oryzanol *In Vitro* by Pepsin and Bile-Pancreatin

The actual digestive process in humans is more complicated than that depicted in this *in vitro* experiment. The capacity of the stomach is 50mL when empty and can expand to 1 to 1.5 liters when filled (Wardlaw and Insel, 1993). The small intestine, composed of the duodenum, the jejunum and the ileum, is altogether approximately 10 feet long. Food stays for 2 to 3 hours in the stomach and 3 to 10 hours in the small intestines. Since γ -oryzanol was the only compound of interest tested in the absence of the complexity of other foods, which require longer time to digest, the scale of the model to study digestibility of γ -oryzanol was reduced but maintained in approximately the same ratio with pepsin, bile and pancreatin solutions as the model used by

Ames *et al.* (1999). For the same reason, the incubation time was also reduced to 2 hours with pepsin and then 2 hours with pancreatin.

The results showed that γ -oryzanol concentrations in both forms of crystalline and micellar solution decreased after peptic and pancreatic digestion compared to incubation with water (Figure 5.1). Interestingly, the susceptibility of these two forms of γ -oryzanol to digestion was not equal. After either peptic or pancreatic digestion, γ -oryzanol recovered from micellar solution was less than that recovered from the crystalline form. It may be explained that the γ -oryzanol in crystalline form, which was insoluble in aqueous solution, was not evenly distributed in the solution to react with pepsin and pancreatin while the micellar form was. Additionally, even though little chemical digestion occurs in the stomach except protein hydrolysis, %recovery of γ -oryzanol decreased more significantly in the form of micelles when incubated with HCl and without pepsin, which may indicate that the effects of both low pH and pepsin on structural integrity of γ -oryzanol exists. The further contribution of pancreatin to γ -oryzanol degradation was also present in both forms of γ -oryzanol, crystalline and micelles, when compared to the γ -oryzanol recoveries after incubation with NaHCO_2 and without pancreatin.

In regard to the pathway of γ -oryzanol degradation during digestion, one could hypothesize that γ -oryzanol might be hydrolyzed by pepsin and the pancreatic enzymes at the ester bond since the chemical structure of γ -oryzanol consists of a terpene alcohol or sterol esterified with a ferulic acid (Figure 2.2(b)). On the HPLC chromatograms of γ -oryzanol after peptic and pancreatin digestion (Figure 5.2), all the γ -oryzanol components decreased and ferulic acid, which if present would also absorb at 330nm with the retention time at 1.5 minutes (Figure 3.1(b)), was not found after either peptic or pancreatic digestion. Also, the further analysis of digestion products by GC/MS showed that the amount of terpene alcohol or sterol portion of γ -

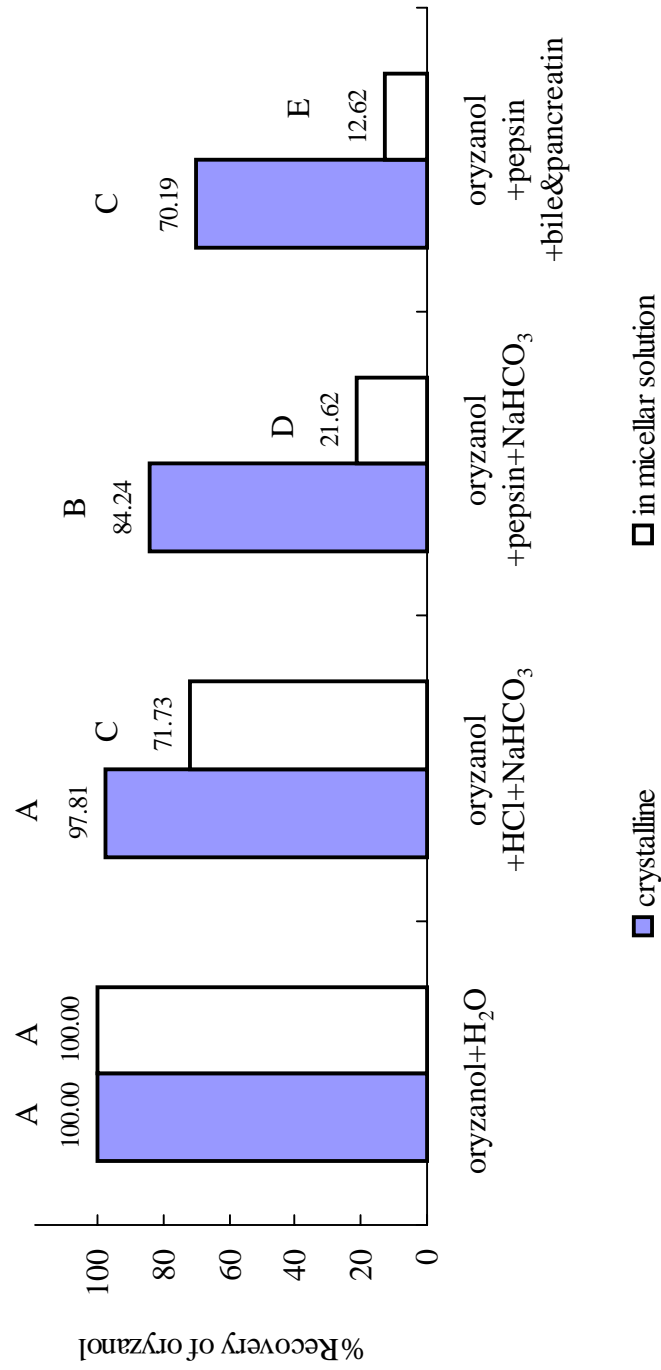


Figure 5.1. *In vitro* digestibility of γ -oryzanol in forms of crystalline or micellar solution incubated with water for 4 hours or HCl or pepsin for 2 hours and then NaHCO₃ or bile and pancreatic mixture for 2 hours at 37°C. (n=2×2 replications. Significant differences ($p<0.05$) are expressed by different letters.)

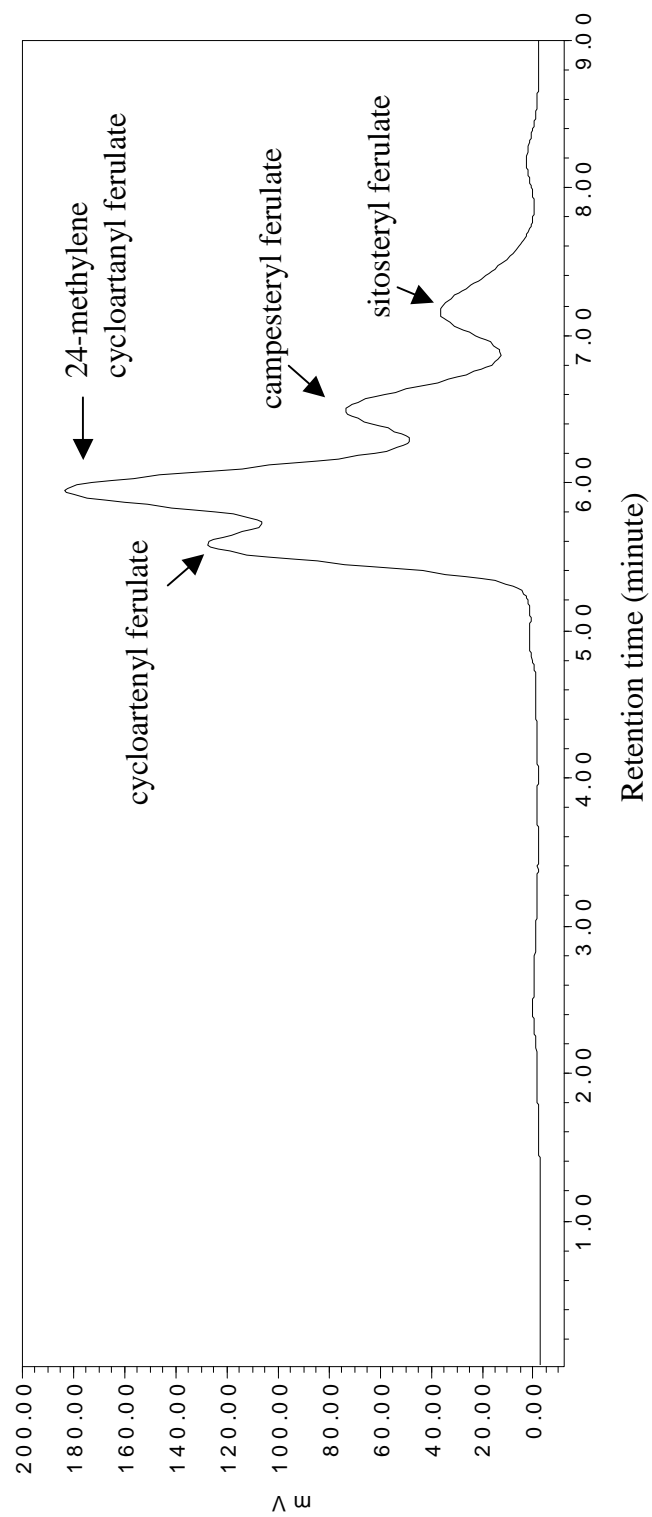


Figure 5.2(a). HPLC chromatogram of 0.1mM γ -oryzanol incubated with H₂O at 37°C for 4 hours

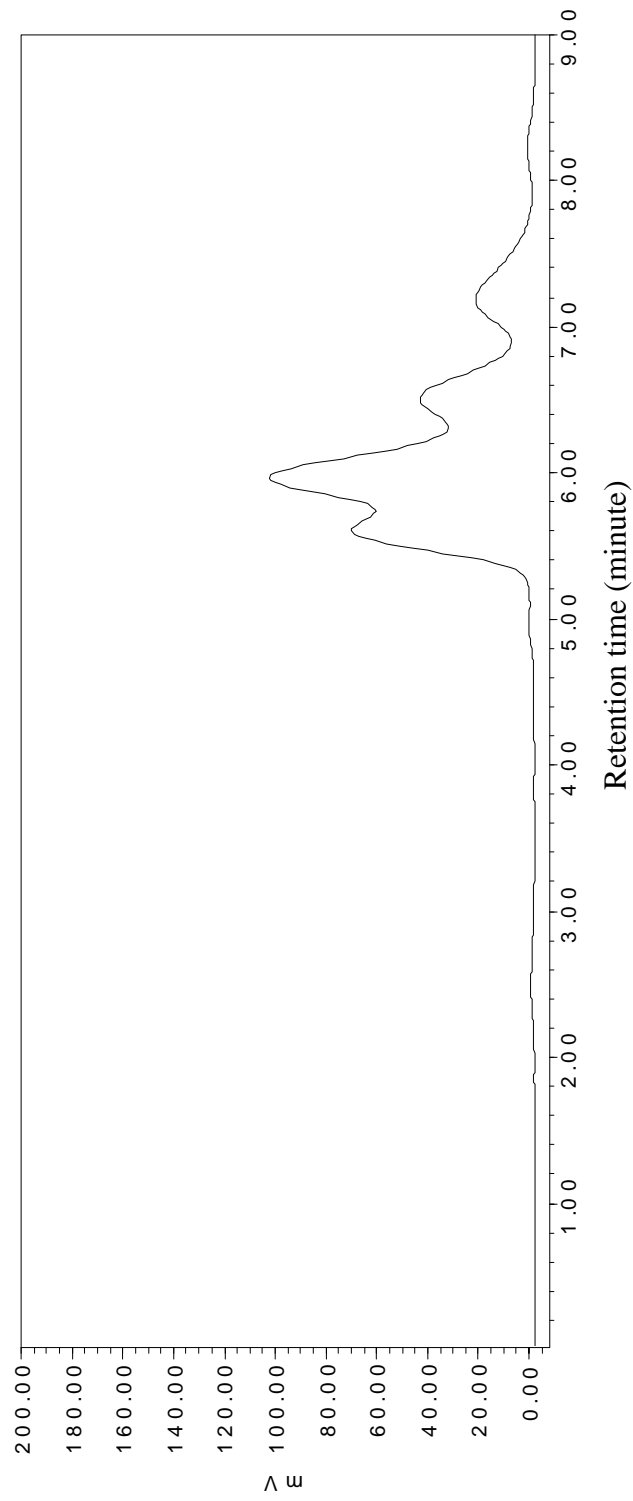


Figure 5.2(b). HPLC chromatogram of 0.1mM γ -oryzanol after peptic (37°C, 2 hours) and pancreatic (37°C, 2 hours) digestion

oryzanol did not increase (Figure 5.3). However, this was inconclusive as to whether or not the breakdown of the ester bond occurred. The digestibility of stigmasterol and ferulic acid was also studied and the results showed that the concentrations of both decreased after digestions (Figure 5.4 and 5.5). Thus, if the ester bond of γ -oryzanol is hydrolyzed, the sterols or terpene alcohols and ferulic acid produced might be further degraded. Otherwise, degradation of γ -oryzanol might undergo a different pathway.

Absorption and metabolism of γ -oryzanol in rats has been studied by Fujiwara *et al.*, 1983. In the first 72 hours after oral administration of ^{14}C -labeled at the C-3 position of ferulic acid, radioactivity analysis showed that 9.8% of the dose (50mg/kg) was excreted in the urine, which was not intact γ -oryzanol. But their results from the experiment of *in situ* intestinal absorption showed that, while 89.4% of the radioactivity remained in the luminal fluid mostly as intact γ -oryzanol suggesting γ -oryzanol was poorly absorbed, more than 80% of the absorbed radioactivity (=0.16% of the dose) was intact γ -oryzanol, transferred into the mesenteric vein and then into portal vein, and 10 to 20% of the absorbed dose was metabolized in the intestinal tissues to yield ferulic acid. However, this *in situ* experiment did not cause the γ -oryzanol to undergo digestions in stomach, duodenum and jejunum, where secreted digestive enzymes could cause γ -oryzanol degradation to occur.

The metabolic fate of ferulic acid has also been studied by Booth *et al.* (1957). In both studies, the proposed urinary metabolites of ferulic acid were dihydroferulic acid, vanillic acid, *m*-coumaric acid, *m*-hydroxyphenylpropionic acid, hippuric acid and *m*-hydroxyhippuric acid (see chemical structures in Figure 2.6). None of these metabolites were found in the digestion products in this study, based on the GC chromatograms and identification by the MS spectrum of the peptic and pancreatic digest products of ferulic acid and γ -oryzanol, assuming that

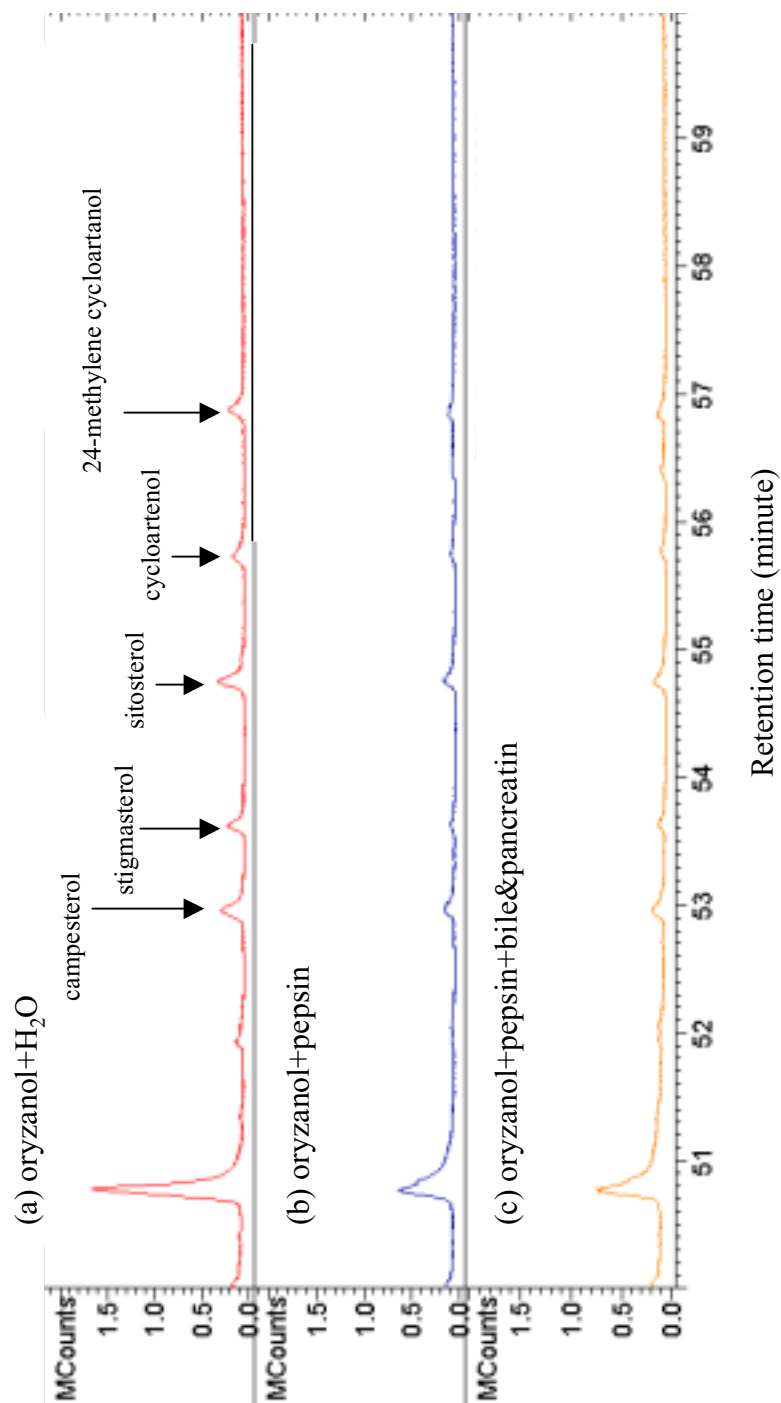


Figure 5.3. GC chromatograms of γ -oryzanol digestion products after incubation with pepsin, 37°C, 2 hours, and then bile-pancreatin mixture, 37°C, 2 hours

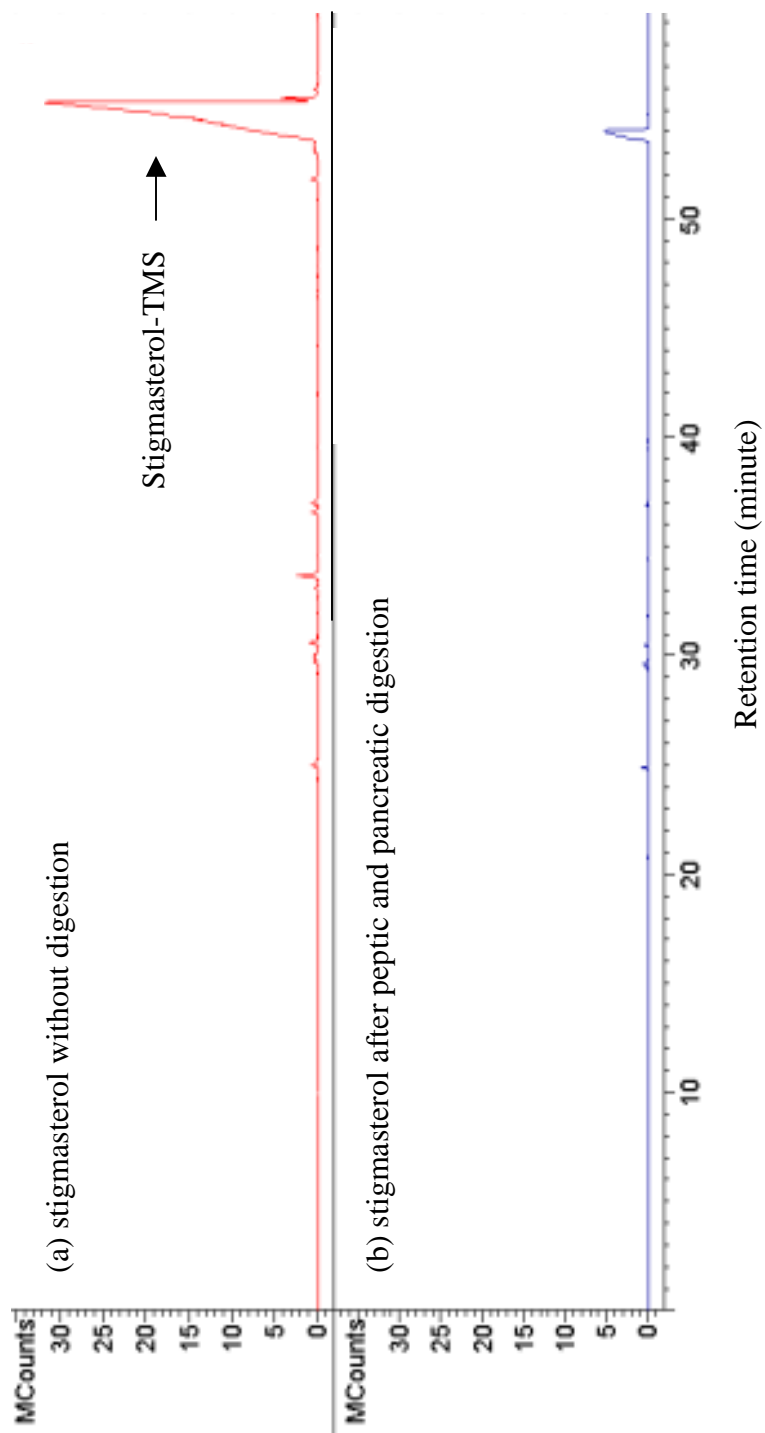


Figure 5.4. GC chromatograms of stigmasterol before and after peptic and pancreatic digestion

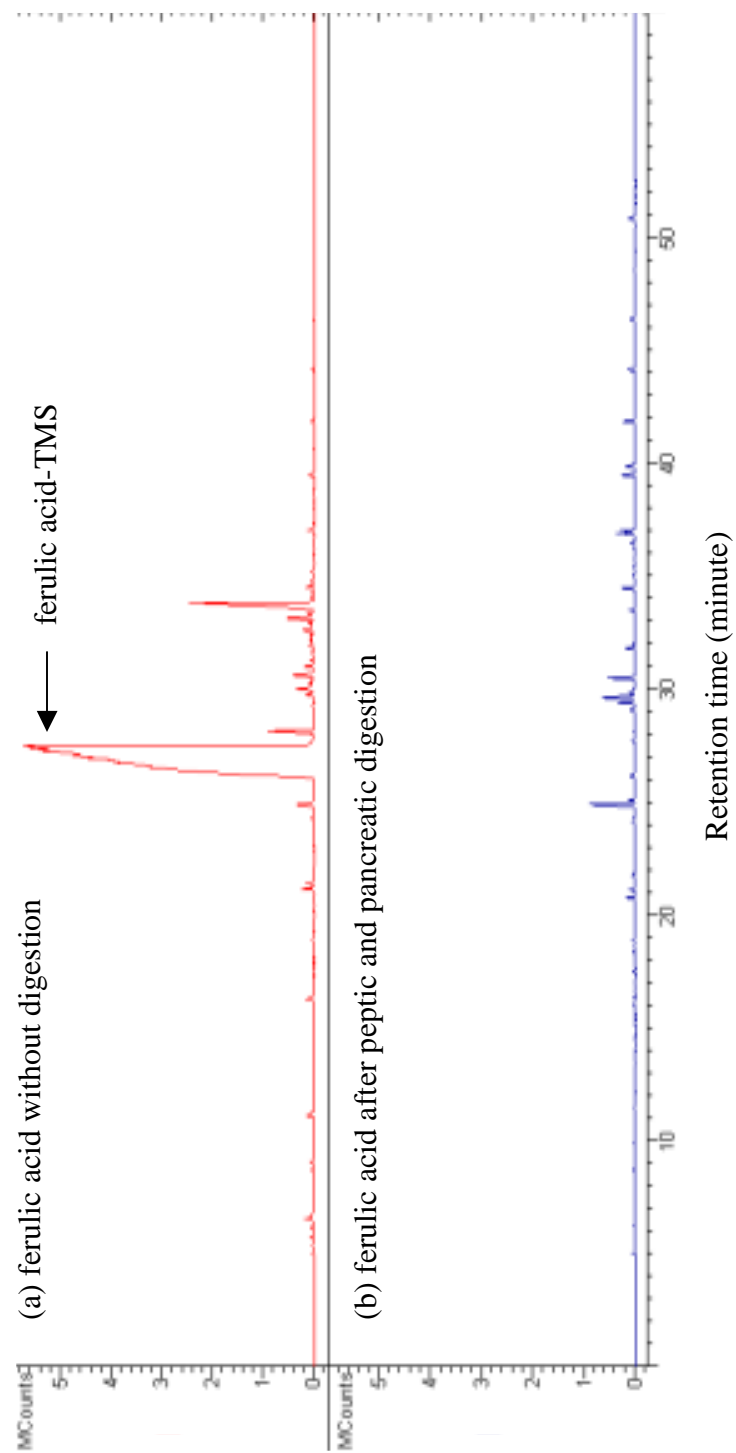


Figure 5.5. GC chromatograms of ferulic acid before and after peptic and pancreatic digestion

hexane/ethyl acetate/isopropanol (2:1:1) plus hexane ($\times 3$) should be efficient to extract ferulic acid and those metabolites if present. However, during that period when the previous studies were done, thin layer chromatography was mostly employed and using R_f values to identify γ -oryzanol and its metabolites may be questionable. More research is required to further delineate the digestibility and metabolism of γ -oryzanol.

Therefore, all the results may imply that γ -oryzanol undergoes a more complicated degradation through the gastrointestinal tract rather than the simple breakdown of the ester bond between terpene alcohol or the sterol portion of γ -oryzanol and ferulic acid.

This experiment focused mainly on chemical digestibility of γ -oryzanol with pepsin and pancreatin and the digestion products yielded from hexane/ethyl acetate/isopropanol (2:1:1) extraction and, therefore, is of a preliminary nature. The effects of gastric and intestinal muscle contraction, the movement of the foods in the gastrointestinal tract, the regulation of the enzyme secretions, the interrelationships with liver and pancreas and the interrelationship between digestive and absorptive processes on digestibility of γ -oryzanol were beyond the scope of this study.

5.3.2 Digestibility of γ -Oryzanol by Pancreatic Enzymes, Cholesterol Esterase, Lipase and Phospholipase A_2

Since it was found that γ -oryzanol concentration decreased after peptic and pancreatic digestion, it was further studied to determine if three of the pancreatic enzymes, CEase, lipase and phospholipase A_2 , were responsible for γ -oryzanol degradation. The results showed that %recovery of γ -oryzanol after incubation with a concentration of CEase higher than 10 μ g protein/mL significantly decreased in a CEase concentration-dependent manner ($p < 0.05$) (Figure

5.6). On the other hand, the %recovery after incubation with lipase or phospholipase A₂ was not in a concentration-dependent manner and the differences were not as pronounced as with CEase.

Furthermore, on the HPLC chromatograms of γ -oryzanol after digestion by pancreatic enzymes (Figure 5.7), campesteryl ferulate and sitosteryl ferulate decreased more obviously, which may imply that they were more susceptible to pancreatic enzyme digestion. The digestion products of γ -oryzanol incubated with CEase were further analyzed by GC/MS and it was found that the amount of campesterol and sitosterol increased (Figure 5.8), which may be because campesteryl ferulate or sitosteryl ferulate was hydrolyzed by CEase at the ester bond to produce campesterol or sitosterol and ferulic acid. However, ferulic acid was not found on any of the HPLC and GC chromatograms.

The reason that campesteryl ferulate and sitosteryl ferulate were more susceptible than the other components of γ -oryzanol to CEase may be due to the similarity of their chemical structures with cholesterol's. Their ring structures are identical with cholesterol (Figure 2.2), as they have neither the dimethyl groups at C-4 position nor the methyl group at C-14 position, while some of the other γ -oryzanol components do. The side chain structures have only an extra methyl group difference at C-24 position for campesteryl ferulate and an extra ethyl group for sitosteryl ferulate. This could also explain that the amount of campesteryl ferulate decreased more after peptic and pancreatic digestion.

Similarly, none of the urinary ferulic acid metabolites, dihydroferulic acid, vanillic acid, *m*-coumaric acid, *m*-hydroxyphenylpropionic acid, hippuric acid and *m*-hydroxyhippuric acid, summarized by Fujiwara *et al.*, 1983, and Booth *et al.*, 1957, were found in this study, according to the GC chromatograms and the identification on the MS spectrum of the CEase, lipase and

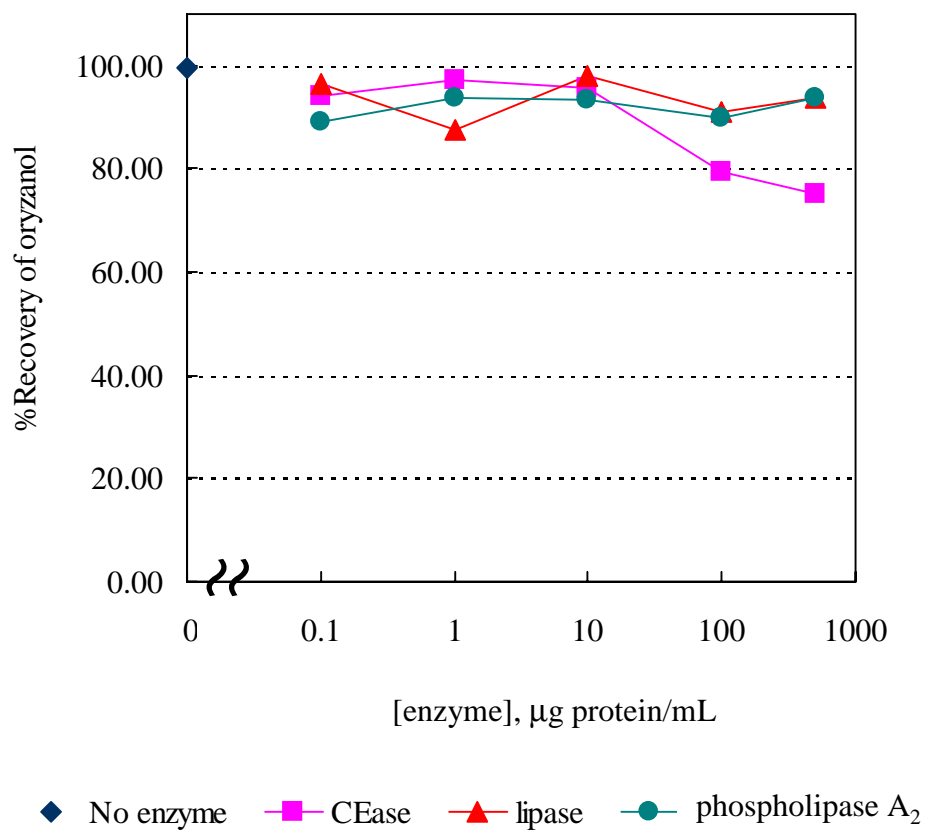


Figure 5.6. Digestibility of γ -oryzanol *in vitro* by cholesterol esterase (CEase), lipase or phospholipase A₂ after incubation for 1.5 hours at 37°C. (n=2×3 replications)

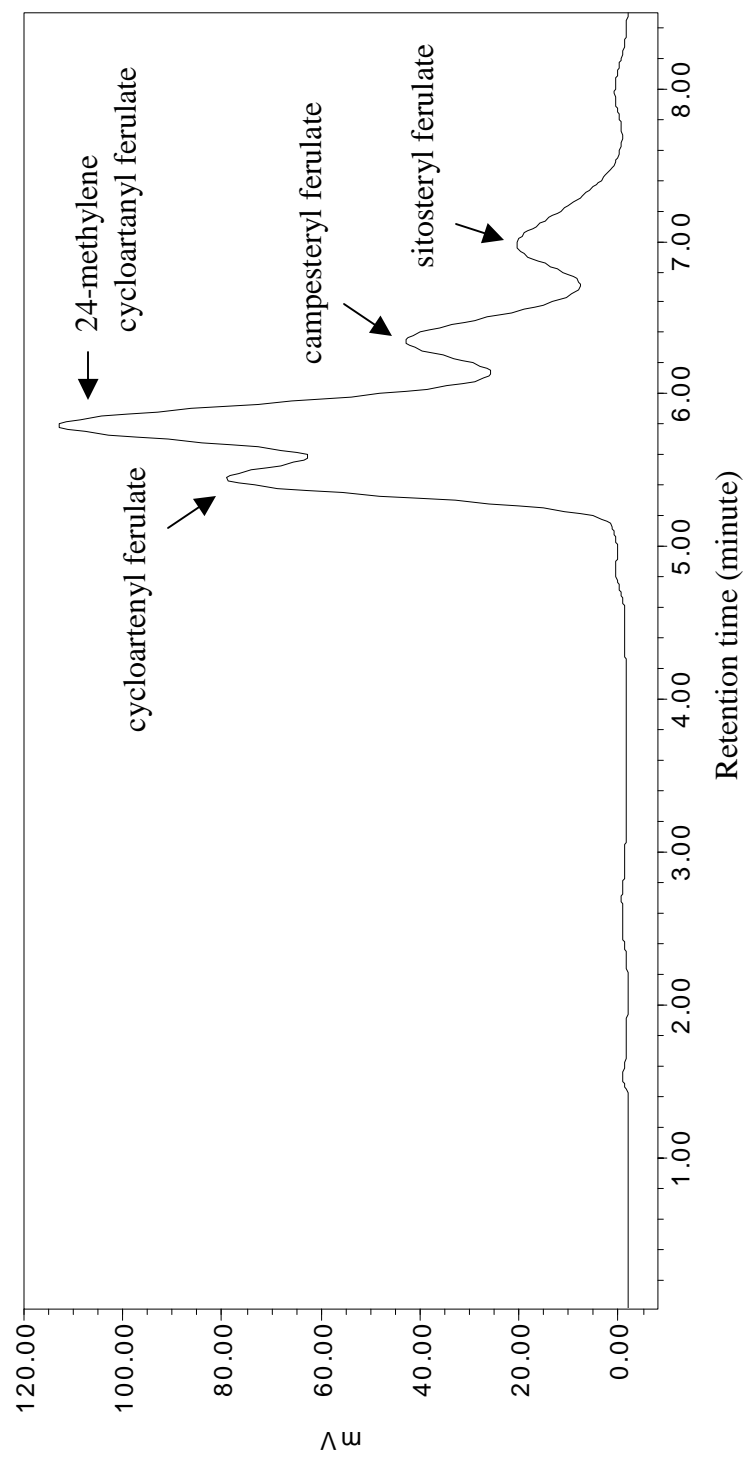


Figure 5.7(a). HPLC chromatogram of 0.1mM γ -oryzanol incubated without enzyme at 37°C for 1.5 hours

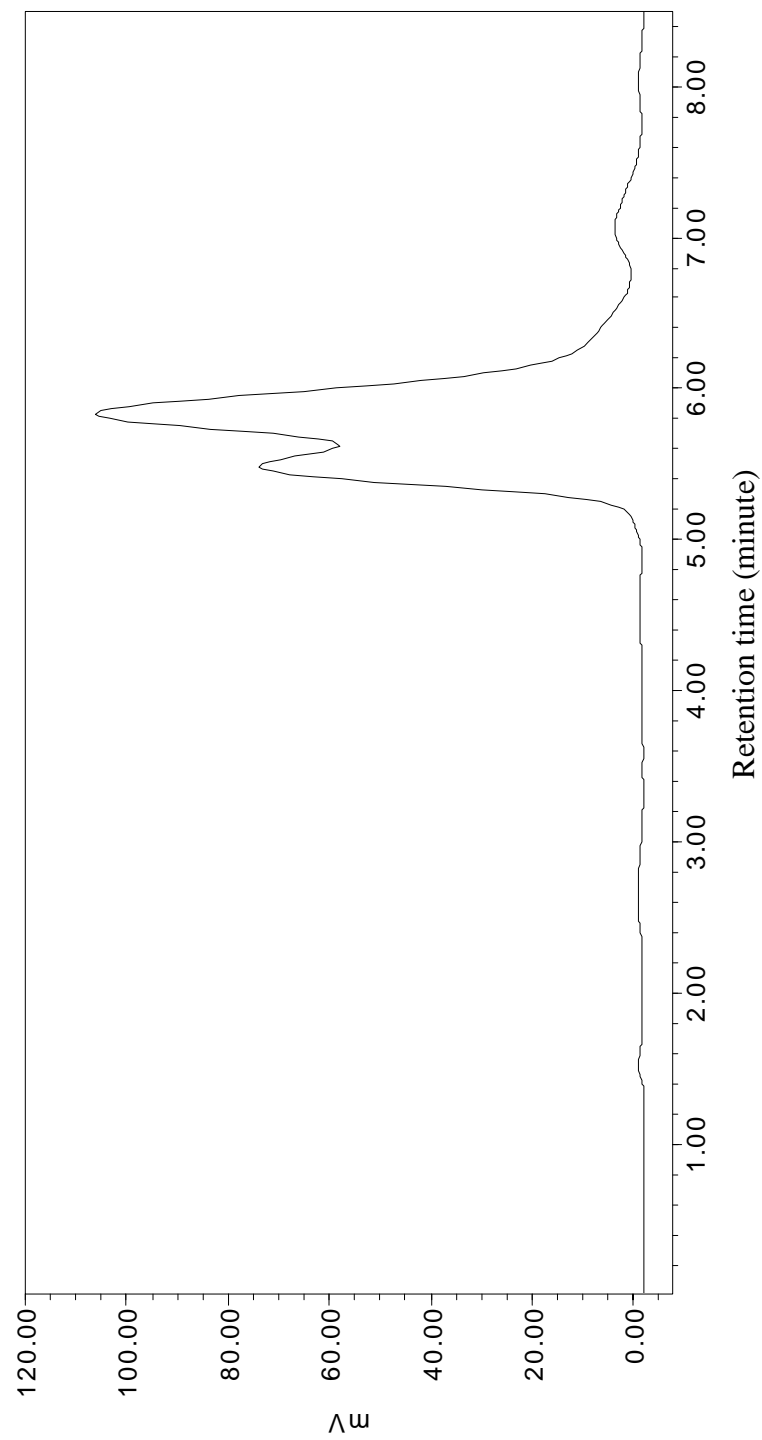


Figure 5.7(b). HPLC chromatogram of 0.1mM γ -oryzanol incubated with 500 μ g protein/mL of cholesterol esterase at 37°C for 1.5 hours

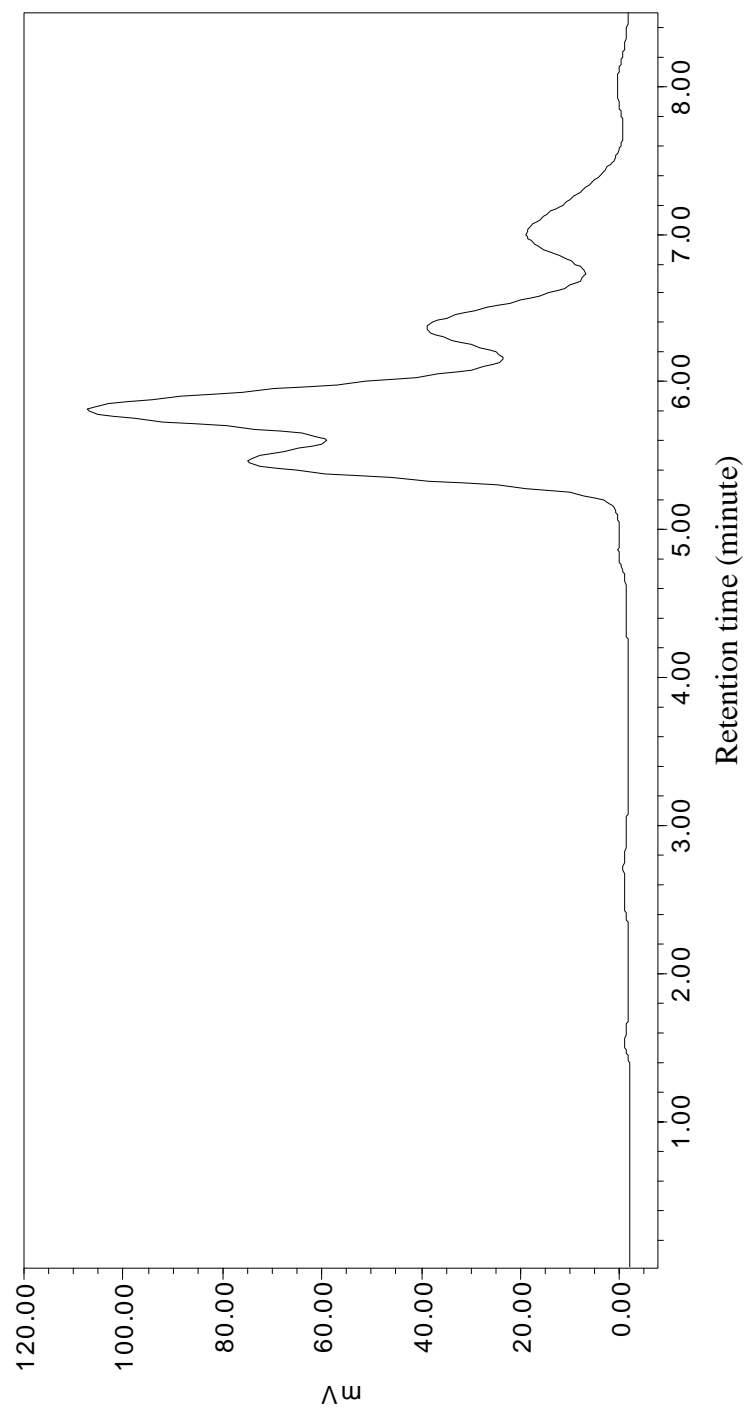


Figure 5.7(c). HPLC chromatogram of 0.1mM γ -oryzanol incubated with 500 μ g protein/mL of lipase at 37°C for 1.5 hours

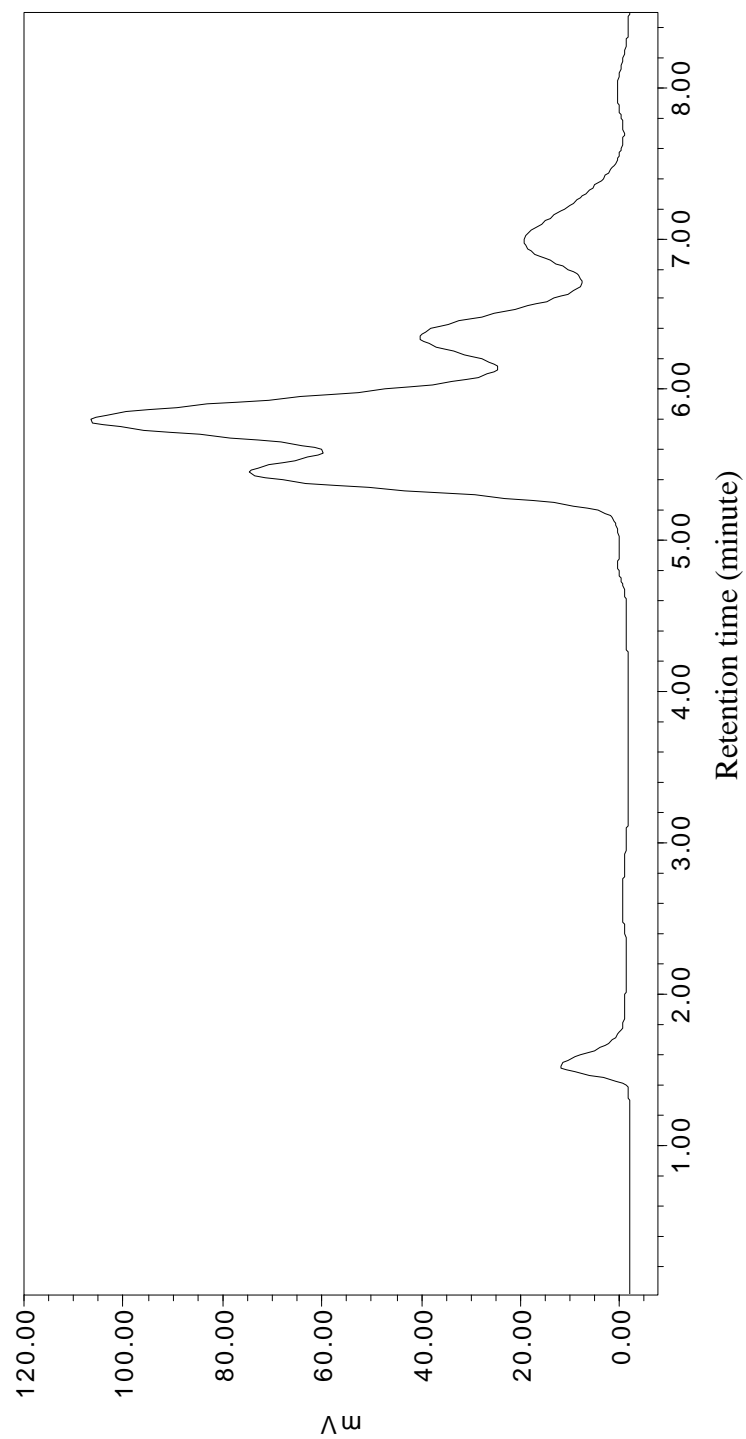


Figure 5.7(d). HPLC chromatogram of 0.1mM γ -oryzanol incubated with 500 μ g protein/mL of phospholipase A₂ at 37°C for 1.5 hours

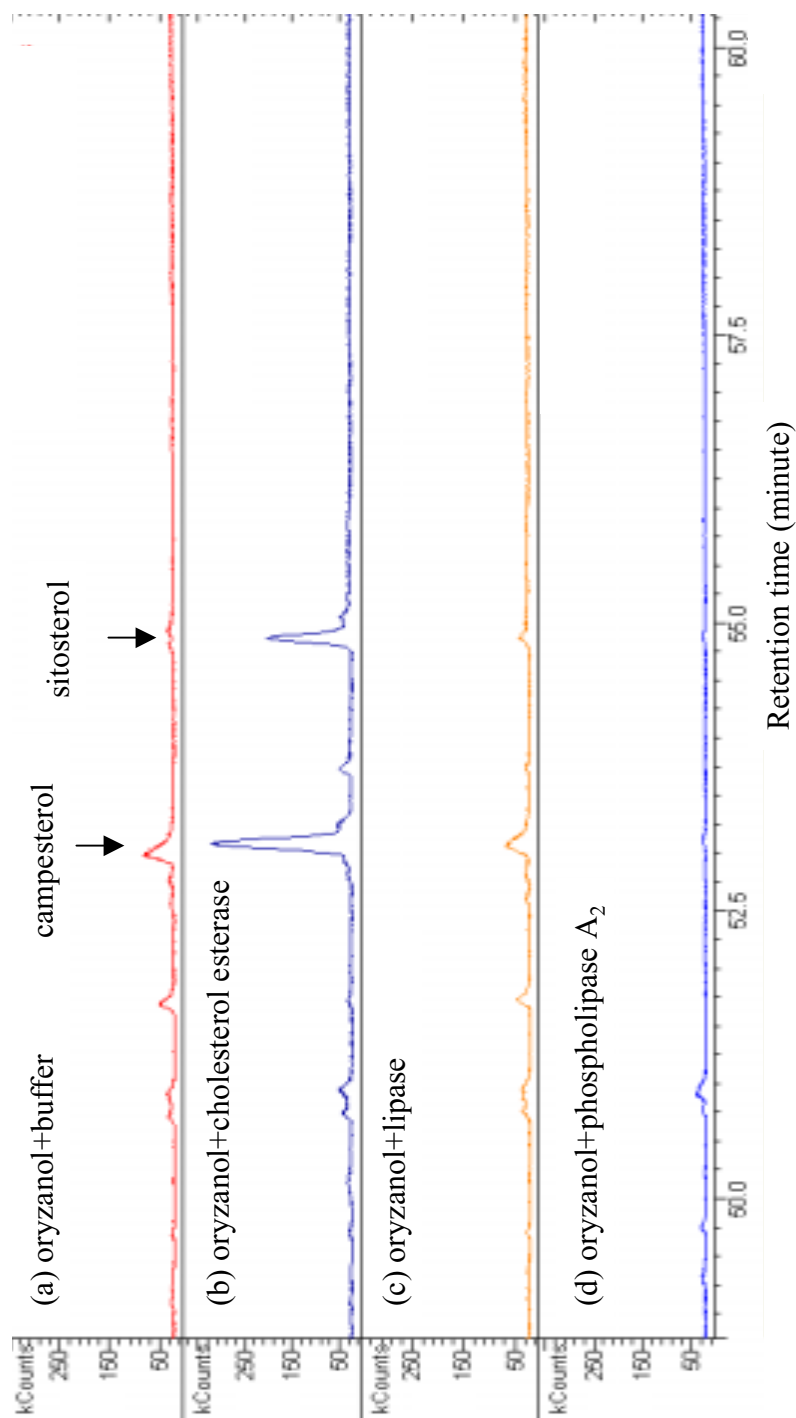


Figure 5.8. GC chromatograms of γ -oryzanol digestion products after incubation with 500 μ g protein/mL of cholesterol esterase, lipase or phospholipase A₂ at 37°C for 1.5 hours

phospholipase A₂ digestion products of γ -oryzanol. This might suggest that ferulic acid was further degraded in a different pathway.

Sterol specificity of CEase has been studied by Swell *et al.* (1954) who summarized that cholesterol, sitosterol and stigmasterol esters with butyric acid were hydrolyzed very rapidly while the hydrolysis of their esters with oleic acid was not as rapid. The order of hydrolytic activity in the first 4 hours was cholesteryl oleate>sitosteryl oleate>stigmasteryl oleate. The results from our study may also provide some additional information about the specificity of CEase, which indicated that the specificity of pancreatic CEase was found not only for cholesterol esters, in this case cholesteryl oleate, but also for campesteryl ferulate and sitosteryl ferulate.

5.3.3 Study of γ -Oryzanol Uptake by Human Intestinal Cells *In Vitro*

First of all, the cytotoxicity results showed that 0.1mM γ -oryzanol in micellar solution did not decrease cellular mitochondrial activity after 2-hour incubation at 37°C, compared to the control, serum-free and transferrin-free culture medium (Figure 5.9). Secondly, since NaOH was used as the cell lysis solution, the effect on γ -oryzanol hydrolysis was also studied by incubation of γ -oryzanol with 1N NaOH without cells at 37°C for 30 minutes. It was found that %recovery of γ -oryzanol was 97.7% and, therefore, cell lysis solution would not cause the degradation of the recovered γ -oryzanol if γ -oryzanol was absorbed. Thirdly, the uptake of intact γ -oryzanol by cells was not detected after 2-hour incubation at 37°C. Longer incubation time was not studied because the cells were vulnerable to the micellar solution, which was required to prepare γ -oryzanol in aqueous environment. However, the same cell model was also used to study cholesterol uptake *in vitro*, and cholesterol uptake by cells was detected under the same conditions. Additionally, when comparing the GC chromatograms of cell extracts after γ -

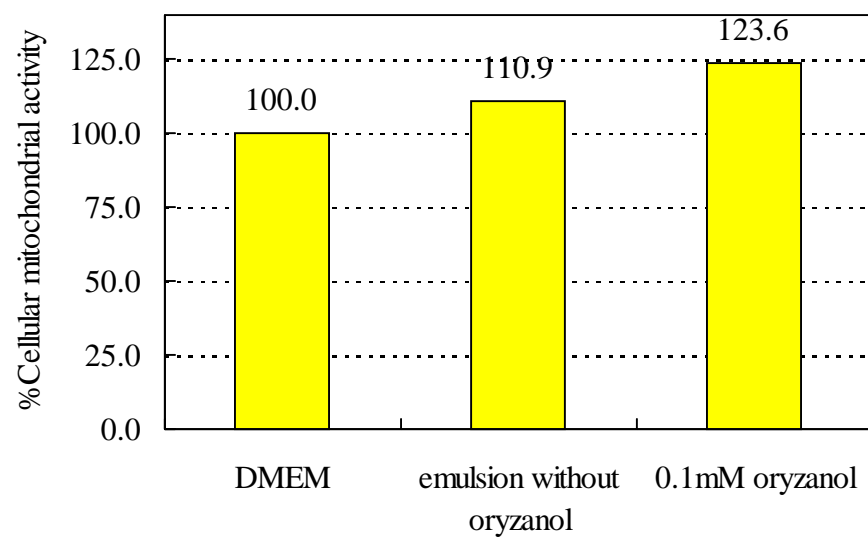


Figure 5.9. Cytotoxicity study of γ -oryzanol micellar solution incubated with human intestinal cells C2BBel at 37°C for 2 hours

oryzanol uptake with those after the control (emulsion without γ -oryzanol) uptake, there were no differences in detected compounds.

Compared to the Fujiwara study on the *in situ* intestinal absorption experiment, they concluded that 89.4% of the dose was found in the luminal fluid 50 minutes after administration, mostly as intact γ -oryzanol, and suggested that γ -oryzanol was poorly absorbed.

5.4 Conclusion

Digestibility of γ -oryzanol focusing on peptic and pancreatic digestions was studied. A cell model using human intestinal cells *in vitro* was also developed to study whether or not γ -oryzanol uptake into cells occurred.

From all the information provided by this study, it may be implied that in the small intestines, the ester bond of γ -oryzanol, especially campesteryl ferulate and sitosteryl ferulate, was broken down by CEase and produced terpene alcohols or sterols and ferulic acid, both of which may be further broken down since they were not found in the peptic and pancreatic digestion products. Even though the degradation of γ -oryzanol, other than steryl ferulates, by CEase was not as noticeable, they may be degraded by other substances in pancreatin since their recoveries also decreased. The degradation of γ -oryzanol in the stomach may undergo a different pathway because sterols or terpene alcohols were not found after peptic digestion. Moreover, γ -oryzanol in micellar solution was degraded more readily than in the crystalline form, proving that micelle formation is necessary for lipid digestion in the gastrointestinal tract since the surface area of dietary lipids in micelles increases and consequently the accessibility of the lipids to the digestive enzymes in aqueous environment increases.

As for the γ -oryzanol uptake by human intestinal cells *in vitro*, intact γ -oryzanol uptake by cells was not detected after 2-hour incubation. In this regard, relative to research that might be

conducted in the future, identification of the digestion products, their cytotoxicity and uptake by human intestinal cells are suggested in order to further understand the digestibility of γ -oryzanol. Furthermore, there has been little information provided relative to biotransformation of γ -oryzanol and its digestion products by microflora in the human colon. This should also be explored in the future since it has been a hot issue to develop functional foods to improve the health of the human colon and to possibly protect against gut-related diseases.

CHAPTER 6. CONCLUSION

In this study, *in vitro* cell models were developed and were used to study the functionality, antioxidant activity and hypocholesterolemic capacity, and digestibility of γ -oryzanol. The cell lines chosen had a fast metabolism and consequently could be used to study the functionality of γ -oryzanol in a relatively rapid manner. The general parameters that are critical and vital when using cell models as tools for functionality experiments, are 1) the emulsion preparation of hydrophobic compounds for cell models, 2) the consistent management of cell culture, and 3) the selection of cell viability detection methods compatible with the cell lines and the test substances.

An *in vitro* cell model using SVEC4-10 mouse lymph endothelial cells was established and proven to be capable of distinguishing antioxidant activity of γ -oryzanol, ferulic acid, the three major components of γ -oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate, as compared with α -tocopherol. tBHP was the oxidizing agent, whose concentration and incubation time combinations were very critical, and the MTT assay was chosen for cell viability determination because it had high response signals and did not interfere with the test substances of interest. Four situations were designed to test whether or not the compounds of interest might protect cells from tBHP oxidation, including 1) being simultaneously present with tBHP and cells, 2) being associated with cells before tBHP oxidation but not present with tBHP, 3) being associated with cells before and during tBHP oxidation, and 4) interacting with tBHP before it reached cells. Generally, γ -oryzanol and ferulic acid were found to possess antioxidant activity in terms of preventing cellular mitochondrial damage due to tBHP oxidation. When γ -oryzanol or α -tocopherol was simultaneously present with cells and tBHP, their antioxidant activity was not significant. When associated with the

SVEC4-10 cells before tBHP oxidation, ferulic acid had higher antioxidant activity than γ -oryzanol and α -tocopherol at the early stage of oxidation (0 to 30 minutes) while γ -oryzanol and α -tocopherol tended to have higher antioxidant activity than ferulic acid at the late stage of oxidation (30 to 60 minutes). The three major components of γ -oryzanol generally had higher antioxidant activity than γ -oryzanol and in the situation when associated with cells before and during tBHP oxidation, they presented more powerful antioxidant activity than α -tocopherol. Among these three major components of γ -oryzanol, 24-methylene cycloartanyl ferulate was found to be relatively more effective. A synergistic antioxidant activity among γ -oryzanol, ferulic acid and α -tocopherol was also found when simultaneously present with tBHP. Since the quantity of γ -oryzanol in rice bran is much greater than vitamin E, γ -oryzanol may play a more important role as an antioxidant to support the potential functional value of rice bran.

Regarding the hypocholesterolemic capacity of γ -oryzanol, this is the first time that the possible mechanism for this γ -oryzanol function has been approached. This study postulated relative to the aspects that γ -oryzanol may affect and then developed a model to investigate the γ -oryzanol effect from each aspect. An *in vitro* cell model using human intestinal cells (C2BBel) accompanied with GC/MS for cholesterol analysis was developed and used to detect cholesterol uptake into cells *in vitro*. The results suggested that the intact γ -oryzanol was poorly absorbed by intestinal cells, therefore, it was speculated that the effect of γ -oryzanol may take place in the lumen of the gastrointestinal tract and the effect was on the availability of cholesterol for uptake into cells. The results suggested activity from three standpoints. 1) γ -Oryzanol may affect the availability of cholesteryl esters by reducing their micellar solubility because the uptake of cholesteryl oleate was not found and the hydrolysis catalyzed by CEase to produce free

cholesterol was necessary. 2) Since the function of CEase to facilitate cholesterol uptake was not found, γ -oryzanol may inhibit the hydrolytic function of CEase, which was essential for the hydrolysis of cholesteryl esters and free cholesterol production. 3) Because it was found that CEase and phospholipase A₂ did not promote the uptake of free cholesterol, γ -oryzanol may affect the availability of free cholesterol for uptake by decreasing its micellar solubility.

Digestibility of γ -oryzanol (in crystalline or in micellar solution) after peptic and pancreatic digestions and the uptake of intact γ -oryzanol into cells were studied by an *in vitro* cell model using human intestinal cells (C2BBe1). γ -Oryzanol was found to be degraded after peptic and pancreatic digestions and the degree of degradation was higher in micellar solution than in crystalline, proving that micelle formation is necessary for lipid digestion in the gastrointestinal tract. The reasons may be relative to increased surface area of dietary lipids in micelles and the consequence of increased accessibility of the lipids to the digestive enzymes in aqueous environment. The effect of three pancreatic enzymes, CEase, lipase and phospholipase A₂, on γ -oryzanol degradation was further studied. The results showed that the ester bond of γ -oryzanol, especially campesterol ferulate and sitosterol ferulate, was broken down by CEase and produced triterpene alcohols or sterols and ferulic acid. However, in the peptic and pancreatic digestion products, triterpene alcohols or sterols and ferulic acid were not found and it may suggest further degradation. The degradation of γ -oryzanol in the stomach may undergo a different pathway because sterols or triterpene alcohols were not found after peptic digestion. Additionally, the uptake of intact γ -oryzanol into cells was not detected, while cholesterol uptake was, after 2-hour incubation.

Finally, more research is required in order to further understand the digestive and metabolic fates of γ -oryzanol, including the degradation reactions, the identification of the

digestion products, the toxicity and uptake of the digestion products into the human intestinal cells, the functionality of the digestion products and interaction with the microflora in human colon.

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APPENDIX I. SAS CODES

A. Antioxidant Activity

```
dm 'log;clear;output;clear;';
options ps=58 ls=98 nodate pageno=1;

data format1combo;
input day column type $ oxtime activity @@;
cards;
1 1 em 0 100.70 1 2 em 0 99.3 1 1 em 15 94.5
1 2 em 15 93.35 ...
5 1 f 30 56.6 5 2 f 30 76.6 5 1 f 45 68.4
5 2 f 45 68.1 5 1 f 60 39.2 5 2 f 60 55.2;
run;
proc sort;
    by day column type oxtime;
run;

proc mixed;
classes day column type oxtime;
model activity=type|oxtime/Ddfm=Satterth;
random day day*type column(day*oxtime);
lsmeans type|oxtime/diff;
run;
quit;
```

B. Cholesterol Uptake

```
dm 'log;clear;output;clear;';
options ps=58 ls=78 nodate pageno=1;

Data cholesteroluptake;
Input trial block trt $ sub uptake @@;
Cards;
1 1 Em 1 18.3745
1 1 C 1 15.7103
...
6 2 CO+ory+CEase 1 12.7126
6 2 CO+ory+CEase 2 14.0195
;

proc mixed;
classes trial block trt;
model uptake=trt/Ddfm=Satterth;
random trial block(trial) trt*block(trial);
lsmeans trt/diff;
run; quit;
```

APPENDIX II. DATA OF ANTIOXIDANT ACTIVITY EXPERIMENTS

(a) Format 1: SVEC4-10 cells were incubated with each test solution and tBHP for 0 to 60 minutes at 37°C (n=2 × 5 replications).

%cellular mitochondrial activity	incubation time (min)				
	0	15	30	45	60
control	100.00 ± 2.88	90.19 ± 3.19	79.93 ± 2.88	65.00 ± 2.88	45.46 ± 2.88
1mM γ -oryzanol	100.00 ± 2.88	88.46 ± 3.19	78.07 ± 2.88	67.89 ± 2.88	47.63 ± 2.88
1mM α -tocopherol	100.00 ± 2.88	92.77 ± 3.19	77.74 ± 2.88	67.54 ± 2.88	49.14 ± 2.88
0.6mM ferulic acid	100.00 ± 2.88	92.34 ± 3.19	80.40 ± 2.88	62.24 ± 2.88	46.49 ± 2.88
0.5mM γ -oryzanol+0.5mM α -tocopherol	100.01 ± 3.79	91.53 ± 3.91	77.80 ± 3.79	65.41 ± 3.79	45.06 ± 3.79
0.5mM γ -oryzanol+0.3mM ferulic acid	100.01 ± 3.79	95.16 ± 3.91	82.58 ± 3.79	60.66 ± 3.79	44.08 ± 3.79
0.5mM α -tocopherol+0.3mM ferulic acid	100.01 ± 3.79	88.11 ± 3.91	78.93 ± 3.79	67.91 ± 3.79	47.33 ± 3.79
0.33mM γ -oryzanol+0.33mM α -tocopherol+0.2mM ferulic acid	100.01 ± 3.79	92.16 ± 3.91	74.70 ± 3.79	69.84 ± 3.79	54.81 ± 3.79

(b) Format 2: Cells were preincubated with each test solution for 22 hours at 37°C. Cells were then washed and incubated with tBHP for 0 to 60 minutes at 37°C (n=2 × 8 replications).

%cellular mitochondrial activity	incubation time (min)				
	0	15	30	45	60
control	97.15 ± 5.10	81.06 ± 6.11	80.26 ± 6.11	63.52 ± 6.11	61.23 ± 4.17
0.5mM γ -oryzanol	97.31 ± 5.64	90.78 ± 7.96	92.76 ± 7.96	80.01 ± 7.96	62.41 ± 4.53
0.5mM α -tocopherol	97.48 ± 5.12	88.55 ± 6.13	87.63 ± 6.13	79.61 ± 6.13	64.48 ± 4.31
0.5mM ferulic acid	95.83 ± 5.12	97.82 ± 6.13	94.47 ± 6.13	73.78 ± 6.13	52.21 ± 4.31
0.25mM γ -oryzanol+0.25mM α -tocopherol	96.91 ± 5.64	93.16 ± 7.96	84.99 ± 7.96	73.39 ± 7.96	65.68 ± 4.53
0.25mM γ -oryzanol+0.25mM ferulic acid	96.97 ± 5.64	98.23 ± 7.96	87.91 ± 7.96	69.06 ± 7.96	61.84 ± 4.53
0.25mM α -tocopherol+0.25mM ferulic acid	96.34 ± 5.64	95.46 ± 7.96	91.54 ± 7.96	74.39 ± 7.96	62.70 ± 4.53
0.2mM γ -oryzanol+0.2mM α -tocopherol+0.2mM ferulic acid	97.14 ± 5.64	90.08 ± 7.96	87.37 ± 7.96	74.61 ± 7.96	60.16 ± 4.53

(c) Format 3: Cells were preincubated with each test solution for 22 hours at 37°C and then with tBHP for 0 to 60 minutes at 37°C (n=2 × 2 replications).

	incubation time (min)				
	0	15	30	45	60
%cellular mitochondrial activity					
control	100.00 ± 8.84	75.73 ± 8.84	67.08 ± 8.84	54.73 ± 8.84	54.50 ± 8.84
0.5mM γ -oryzanol	100.00 ± 8.84	94.63 ± 8.84	88.15 ± 8.84	78.90 ± 8.84	81.80 ± 8.84
0.5mM α -tocopherol	100.00 ± 8.84	93.80 ± 8.84	89.25 ± 8.84	83.10 ± 8.84	74.63 ± 8.84
0.5mM ferulic acid	100.00 ± 8.84	107.53 ± 8.84	94.88 ± 8.84	72.75 ± 8.84	59.13 ± 8.84

(d) Format 4: Each test solution was preincubated with tBHP in vials for 22 hours at 37°C and then cells were incubated with each mixture for 0 to 60 minutes at 37°C (n=2 × 8 replications).

	incubation time (min)				
	0	15	30	45	60
%cellular mitochondrial activity					
control	97.11 ± 5.57	80.51 ± 6.63	71.63 ± 6.55	57.28 ± 6.55	33.30 ± 4.70
1mM γ -oryzanol	96.48 ± 5.59	81.78 ± 6.57	69.83 ± 6.57	56.83 ± 6.57	34.10 ± 4.76
1mM α -tocopherol	99.02 ± 5.62	84.21 ± 6.57	72.28 ± 6.57	68.11 ± 6.57	38.94 ± 4.75
0.6mM ferulic acid	100.76 ± 5.59	84.49 ± 6.65	74.40 ± 6.64	64.02 ± 6.57	32.00 ± 4.75

(e) Format 2 with each test solution at different dilutions including the three major components of γ -oryzanol. tBHP oxidation was 60 minutes at 37°C (n=2 \times 8 replications).

	%cellular mitochondrial activity	concentration (mM)				
		0	0.05	0.1	0.25	0.5
	control	61.73 \pm 5.88				
	0.5mM γ -oryzanol		58.21 \pm 6.34	61.09 \pm 6.34	62.04 \pm 6.34	62.90 \pm 6.05
	0.5mM α -tocopherol		65.52 \pm 6.26	66.32 \pm 6.26	67.92 \pm 6.26	64.66 \pm 5.91
	0.5mM ferulic acid		65.63 \pm 6.26	64.24 \pm 6.26	64.59 \pm 6.26	52.39 \pm 5.26
	0.25mM γ -oryzanol+0.25mM α -tocopherol		65.12 \pm 6.34	66.17 \pm 6.34	66.73 \pm 6.34	66.17 \pm 6.05
	0.25mM γ -oryzanol+0.25mM ferulic acid		61.01 \pm 6.34	61.35 \pm 6.34	61.15 \pm 6.34	62.34 \pm 6.05
	0.25mM α -tocopherol+0.25mM ferulic acid		64.37 \pm 6.34	64.07 \pm 6.34	64.29 \pm 6.34	64.02 \pm 6.05
	0.2mM γ -oryzanol+0.2mM α -tocopherol+0.2mM ferulic acid		64.11 \pm 6.34	65.16 \pm 6.34	63.78 \pm 6.34	60.65 \pm 6.05
	0.5mM cycloartenyl ferulate		64.28 \pm 6.39	63.43 \pm 6.39	65.62 \pm 6.39	63.82 \pm 6.13
	0.5mM 24-methylene cycloartanyl ferulate		59.54 \pm 6.39	59.52 \pm 6.39	63.32 \pm 6.39	68.29 \pm 6.13
	0.5mM campesterol ferulate		59.29 \pm 6.39	60.51 \pm 6.39	61.76 \pm 6.39	62.77 \pm 6.13

APPENDIX III. DATA OF CHOLESTEROL UPTAKE EXPERIMENTS

- (a) Effect of γ -oryzanol on micellar solubility of cholesterol and cholesteryl oleate (n=2 \times 2 replications).

%recovery of cholesterol, cholesteryl oleate or oryzanol in supernatant

cholesterol + ethanol	100.00 \pm 9.52
cholesterol + oryzanol	91.50 \pm 9.52
cholesteryl oleate + ethanol	100.00 \pm 9.52
cholesteryl oleate + oryzanol	86.08 \pm 9.52
oryzanol + control emulsion	100.00 \pm 0.50
oryzanol + cholesterol	16.78 \pm 0.50
oryzanol + cholesteryl oleate	4.82 \pm 0.50

- (b) Effect of γ -oryzanol preincubated with cholesterol for 0, 1, 2 or 6 hours on cholesterol uptake into human intestinal cells *in vitro*, 2 hour, 37°C. (n=2 \times 3 replications).

cholesterol uptake (μ mol cholesterol/mg cell protein)

cholesterol	18.52 \pm 3.82
preincubation of cholesterol for 6 hours	16.77 \pm 3.61
cholesterol + oryzanol	17.82 \pm 2.65
preincubation of cholesterol + oryzanol for 1 hour	17.33 \pm 3.18
preincubation of cholesterol + oryzanol for 2 hours	16.16 \pm 2.77
preincubation of cholesterol + oryzanol for 6 hours	14.05 \pm 3.02
control emulsion	15.58 \pm 2.36

- (c) Effect of γ -oryzanol preincubated with cholesteryl oleate for 0 or 1 hour before cholesterol esterase-facilitated hydrolysis on cholesterol uptake into human intestinal cells *in vitro*, 2 hour, 37°C. (n=2 \times 3 replications).

cholesterol uptake (μ mol cholesterol/mg cell protein)

cholesterol	18.52 \pm 3.82
cholesteryl oleate + CEase	18.97 \pm 2.51
cholesteryl oleate + oryzanol + CEase	16.01 \pm 2.77
preincubation of (cholesteryl oleate) for 1 hour + CEase	19.14 \pm 3.18
preincubation of (cholesteryl oleate + oryzanol) for 1 hour + CEase	14.89 \pm 3.18
cholesteryl oleate	16.81 \pm 2.37
control emulsion	15.58 \pm 2.36

VITA

Chih-chun Huang was born and grew up in Taiwan, a wonderful country. She is proud to be a Taiwanese.

She attended the Department of Animal Science at National Taiwan University from 1990 to 1994.

Later she came to the United States and was admitted to the Department of Food Science at Louisiana State University, where she received her master degree. She stayed and continued to study and now is a candidate for the doctoral degree to be awarded in 2003. She will return to her country for her family and hopefully contribute what she learned in the United States.